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THE EFFECT ON THE OFFSPRING OF INTOXICATING THE MALE PARENT AND THE TRANSMISSION OF THE DEFECTS TO SUBSEQUENT GENERATIONS

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IT is a thoroughly demonstrated fact that the fertilized egg may be so treated or modified during development as to cause it to give rise to abnormal embryos of definite types. Experiments on the unfertilized egg or female germ cell are not nearly so numerous, are more difficult to perform, and the results are not so decided. The treatment of the male germ cell, or spermatozoon, so as to modify it and to cause a modified development of the egg which it subsequently fertilizes, is an experiment which has rarely been performed with success. In the present communication we wish to consider rather briefly the various methods of treating or modifying the spermatozoon or male germ cell and the result of this modification on the embryo which arises when such a spermatozoon fertilizes an egg. In order to fully appreciate the results obtained by experiments on the sperm it becomes necessary to refer from time to time to the effects derived when the egg is similarly treated.

Since the positive literature bearing on the artificial modification of the sperm is not extensive, I shall first consider it in a general way, and devote the latter part of the paper to the results obtained in a set of experiments which have been conducted on guinea-pigs for the last few years.

The spermatozoon is more difficult to treat experimentally than is the ovum, on account of the fact that the treatment so often renders it inactive or cripples it in such a way that it is unable to penetrate and to fertilize an egg. The spermatozoon, although apparently delicate, is more or less resistant, so that a mild treatment gives no effect. The difference between the upper non-effective dose of treatment and the fatal or ''paralyzing'' dose is slight, yet it is the precise treatment between these two points which yields results.

Little has been done in treating chemically the spermatozoa of invertebrates, though some of the hybridization experiments furnish indirect evidence as to what might occur. Herbst's experiment of starting the development of an egg by parthenogenesis and then fertilizing one blastomere with a foreign spermatozoon offers splendid opportunity for investigating the influence of strange substances introduced by the sperm. The spermatozoa of the sea urchin have been subjected to the action of radium emanations by Günther Hertwig, who found that after intensive treatment for several hours such spermatozoa subsequently disturb the processes of division in the eggs which they have fertilized. Paula Hertwig found that fertilized ascaris eggs treated several hours with radium preparations gave pathological division figures; the chromatin bodies showed a tendency to break down and disintegrate; division was slow and the karyokinetic figures were finally completely deranged.

Investigations on vertebrates have been more extensive. The most beautiful results have been obtained on fish and amphibians through the radium experiments of Oscar Hertwig, his son Günther and his daughter Paula. The outcome of these experiments is so striking and is of such serious importance that I shall dwell somewhat upon their significance. About three years ago O. Hertwig published the results of his first radium experiments in the *Proceedings of the Prussian Academy of Sciences.* At that time he showed that when the unfertilized eggs of a frog are treated for a certain time with radium rays, these eggs, after being fertilized by normal spermatozoa, develop abnormally. Hertwig also found that when the milt or semen of the frog was exposed to the action of radium for a certain time the spermatozoa swimming in it were injured. When eggs were fertilized by these sperm they always developed abnormally. The general type of the abnormalities was the same whether the eggs alone had been treated before fertilization or whether the sperm was treated. When both eggs and sperm were treated the developmental modifications were still more pronounced, though Hertwig claims that the deformities were of a similar type to those which occurred after treating only one of the cells.

Since that time Oscar Hertwig with his son and daughter have extended and analyzed these experiments in a comprehensive fashion. When the sperm of a number of amphibians, frog, toad and salamander, are exposed for five minutes to 5.3 mg. of radium bromide, normal eggs fertilized by such sperm give defective embryos, the defects being generally shown by the central nervous system. They are really of the nature of developmental arrests or degeneration.

If the spermatozoa are exposed for fifteen minutes the effects on development are still more marked. When, however, the sperm in salt solutions are treated intensively for 2 and 3 hours between two mesothorium capsules, the results are most surprising. In one experiment almost all the eggs fertilized by such sperm went *normally,* and in other experiments they went almost normal but slow, yet they were extraordinarily better than eggs that were fertilized by sperm that had been treated for only five minutes. After three weeks the radium larvæ were still behind the control. Hertwig concluded that the spermatozoon had been so injured by the intensive treatment that it could no longer take part in development.

although it could penetrate the egg and cause it to develop parthenogenetically.

A number of important experiments were tried to test the correctness of this conclusion. The most striking of these were those performed by Günther Hertwig in crossing different species.

It is well known that the sperm of the frog will fertilize the egg of the toad or of another species of frog, but the resulting development proceeds for only a short time and the egg usually dies in the blastulæ stage. Günther Hertwig decided that if the influence of the sperm in development was really destroyed by the intensive radium treatments then such a spermatozoon would merely serve as a parthenogenetic agent and the egg should develop in a normal manner, yet be parthenogenetic. He used the eggs of *Bufo vulgaris* (the common toad) and of *Rana viridis* (the green water frog), and the sperm of *Rana fusca*. He ran two sets of each kind of eggs; one set was fertilized by normal *R. fusca* sperm; the other by sperm which had been treated for two or three hours between two capsules of mesothorium. The eggs fertilized by normal sperm did not develop beyond the blastular stage as was expected, while those fertilized with the radiumized sperm developed about normally and hatched from the jelly and gave rise to swimming tadpoles. O. Hertwig repeated this experiment by crossing the eggs of *Triton vulgaris* with the sperm of *Salamandra maculata*. The sperm were treated for 2¼ hours between two strong mesothorium preparations. Poll had found that this cross proceeded only as far as the blastular stage and Hertwig confirmed this.

When, however, the semen was treated for 2¼ hours a different result was obtained. Many eggs failed to become fertilized, some showed polyspermy, and only six went normally. The chromatin of the sperm was thought to be destroyed and the eggs went by parthenogenesis. Loeb has found in the remarkably wide crosses he has made on invertebrates and vertebrates that the products of part of the foreign sperm finally act as a poison and cause the eggs to develop abnormally. The types of monsters in these crosses are similar to those produced by treating the eggs with chemical poisons. In many cases these sperm take no part in development but initiate the process by serving as a parthenogenetic agent. Hertwig takes the same position, and further finds, that when the foreign sperm is treated with radium the injurious substance contained in it is killed or destroyed so that the spermatozoon initiates development by parthenogenesis without later causing the development to be abnormal. Bataillon's method of sticking eggs with fine platinum needles to give artificial parthenogenesis is similar, Hertwig thinks, to the use of sperm intensively treated with radium. The treated spermatozoon plays the rôle of the platinum needle in Bataillon's experiment. The male chromatin can no longer combine with the female chromatin, there is no amphimixis. Bataillon, by his sticking method, obtained from 10,000 *R. fusca* eggs only 120 hatched tadpoles, and but three metamorphosed, while in some of Hertwig's radium experiments almost all hatched from the jelly.

The radium experiments of Hertwig give us the first method of artificial parthenogenesis which offers promise for use with mammals. Hertwig suggests that since artificial fertilization is possible in many mammals, one might fertilize with semen which had been intensively treated with radium so that the chromatin was destroyed, and with such sperm artificial parthenogenesis in mammals could be accomplished. Two years before Hertwig made this suggestion Dr. Congdon was trying the effects of radium on the spermatozoa of mice and rats in the anatomical laboratory at Cornell and is now continuing these experiments in the anatomical laboratory at Stanford University; up to now he has not succeeded in obtaining fertilization with the modified spermatozoa, though of course much experimentation is necessary in order to establish the proper intensity of the treatment.

These experiments of Hertwig also afford interesting data as to the nature and importance of the part played by the chromatin in development. The cells of the embryos which resulted from eggs fertilized by intensively treated sperm were found by O. Hertwig, P. Hertwig, and Poll, to contain the reduced number of chromosomes showing that the paternal chromatin had been destroyed by the treatment. Günther Hertwig found the nucleus volume in radium larvæ to be one half the size of the nucleus in the control; he measured the mass of nuclei of nerve cells, liver cells, blood corpuscles, embryonic muscle cells, etc. The entire larva was smaller. P. Hertwig found the male pronucleus derived from intensively treated sperm to be modified in the first and second divisions of the frog's egg and Opperman found the same in the trout. O. Hertwig found in *Triton* eggs that the intensively radiumized male chromatin took no part in the developmental process and the soma cells contained one half the chromosome number. The male chromosome set falls out of the development and the soma nuclei contain only the female set.

Finally, Hertwig obtained another most striking result which may be mentioned, although it is not entirely in line with the present subject. When eggs instead of the spermatozoa were subjected to intensive treatments of 2 to 5 hours with radium, the chromatin of the female pronucleus was found to be broken down and destroyed. If eggs, after such intensive treatment, were fertilized by normal sperm, it was found that they developed almost normally, although when eggs were treated from 15 minutes to $\frac{1}{2}$ hour they always developed abnormally though fertilized with normal spermatozoa. Hertwig, therefore, concludes that the intensively treated eggs fertilized by normal sperm develop by the process of merogony; that is, the egg nucleus being destroyed by the treatment, the sperm nucleus enters the egg and causes development to proceed in the same way that the female pronucleus acts in parthenogenesis. Only one set of chromosomes, either the paternal or maternal, is necessary for development of the egg.

During the summer of 1912 I treated the spermatozoa of fish with various salts and organic substances with negative results. When the treatment was sufficiently strong to affect the spermatozoa it rendered them incapable of fertilizing the eggs. A method could no doubt be devised for modifying fish spermatozoa with various chemicals and of course radium does modify the fish sperm as Opperman found.

Only a few experiments have been performed in attempting to modify the offspring of birds by injuring the male. Todde found that the offspring from alcoholized roosters were not quite normal and that the roosters did not succeed as well as usual in fertilizing eggs. Lustig's experiments showed that by inoculating fowls with abrin the offspring were rendered less resistant to inoculations of abrin than were control animals of the same age. This result followed the inoculations of either parent, the male as well as the female.

A more extensive literature bears upon the actions of poisons on the male germ cells of mammals, though most of the cases are uncontrolled observations. The treatment of the germ cells of mammals is a more complex proposition than the experiments on those lower forms in which the fertilization is external and where, for this reason, the eggs and spermatozoa may be treated directly. In mammals the stimulus must be applied through the animal body and the case is thus complicated since it is often impossible to differentiate between the direct action of the substance applied and the secondary effects due to the responses of the parental body to the treatment. With certain treatments, however, the case is not so complex as would appear at first sight, since the substances may pass into the blood stream and the lymph and act directly on the germ cells just as they do on other tissues and cells of the body.

In experiments to modify the germ cells of mammals the first proposition becomes then, to determine whether the substances used reach the germ cells directly. One of the best substances for such experiments is alcohol, since its action and distribution in the body has been largely studied and since it acts so decidedly to modify the developmental processes, as many workers have found on invertebrates, and as I have shown by treating fish eggs with this substance.

It is a well known and generally accepted fact that alcohol does cause changes and degeneration in many of the tissues of animals and man. The question arises, how, then, can the reproductive tissues, the ova and spermatozoa escape? Nicloux and Renault have found that alcohol has a decided affinity for the reproductive glands. In the testicular tissues and the seminal fluid an amount of alcohol is soon present which almost equals that in the blood of an individual having recently taken alcohol. The proportion of alcohol in the testis as compared with that in the blood was as 2 to 3, and in the ovary of female mammals as 3 to 5. From these observations it must follow that alcohol may act directly on the ripe spermatozoon shortly before it fertilizes the egg, and if this substance injuriously affects the germ cells, then one should expect to find an indication of the injury in the resulting development as Hertwig has found from his radium treated spermatozoa.

There are a number of observations on human beings bearing on this point, though they probably all need confirmation by experimentation on lower mammals. Lippich claims to have observed 97 children resulting from conception during intoxication. Only 14 of these were without noticeable defects. Twenty-eight were scrofulous, three had "weak lungs," three showed different atrophic conditions, one watery brain, four feeble-minded, etc. Sullivan reported seven fairly authentic cases of drunkenness during conception; six of the offspring died in convulsions after a few months, and the seventh was stillborn.

Rösch was the first, in 1837, to study the reproductive glands of alcoholics and found degeneration of the testicles. Lancereaux described a parenchymatous degeneration of the seminal canals. Simmonds (1898) found azoospermie (spermatozoa without tails) in 60 per cent. of cases of chronic alcoholism; 5 per cent. of these men were sterile. Kyrle reported three cases of total atrophy of the testicular parenchyma in which death had resulted from cirrhosis of the liver due to alcohol. He attributed the atrophy of the testicle to the cirrhosis of the liver and not to chronic alcoholism.

Bertholet (1909) has made an extensive examination of the influence of alcohol on the histological structure of the germ glands, particularly on the testicles of chronic alcoholics. He found testicular atrophy in alcoholics with no cirrhosis of the liver. Bertholet observed partial atrophy of the testicles in the majority of 75 chronic alcoholics. The men died between the ages of 24 and 57 years, the greatest mortality being between 30 and 50 years. In 37 cases, excluding syphilitics, a microscopical examination showed a more or less diffuse atrophy of the testicular parenchyma and a sclerosis of the interstitial connective tissue. The canals were reduced in size and their lumina obliterated. Spermatogonia were atrophic. It was generally impossible to differentiate spermatocytes or spermatids. There were no dividing cells and no spermatozoa. These conditions with slight variations were found in 24 cases. Such atrophic structures were present in one drinker only 29 years old. In 4 cases of cirrhosis of the liver the testicular atrophy had not progressed very far and spermatozoa were still present.

The extreme conditions of atrophy of the testicles were only found in alcoholics. Observing the testicles of nonalcoholics that had died of various chronic illnesses, such as tuberculosis, no atrophy of the testicles or thickening of the membrana propria was found. Two old men of 70 and 91 years still possessed spermatozoa in the canals.

Bertholet concluded that the atrophy he observed was not due to old age, cirrhosis of the liver, or other systemic conditions, but to the effects of chronic alcoholism on the reproductive glands. Weichselbaum has confirmed the observations of Bertholet.

It is certain, however, that the chronic alcoholic is not so often rendered sterile as Bertholet's study would lead one to believe. It is not rare to find alcoholics with large families. My experiments on mammals may not be of sufficient duration at the present time, yet I have male guinea-pigs that have been almost intoxicated on alcohol once per day for six days a week for a period of 32 months, which are still good breeders. Thirty-two months of a guinea-pig's existence is proportionately equal to a good fraction of a human life. A number of these animals have been killed and their testicles examined microscopically and found to be normal. In some cases where the male had failed to succeed in impregnating the female for several times, one of his testicles was removed and studied microscopically; the testicle was found to be normal and the male later gave offspring by other females. Ovaries have been similarly examined and in no case has the alcoholic treatment caused a visible structural change in the reproductive glands. The actual physiological proof of the efficiency of the organs is shown by the ability of the animals to reproduce. Although there is no visible structural change in the germ cells, nevertheless, they have been modified by the treatment to an extent sufficient to cause them in most cases to give rise to defective embryos or weakened individuals which die soon after birth.

Nicloux has carefully demonstrated on dogs and guinea-pigs the passage of alcohol from the blood of the mother into the tissues of the embryo. After a short time the amount of alcohol in the blood of the fetus is about equal to that in the blood of the mother, while there is really slightly more alcohol in a given weight of the tissues of the fetus than is to be found in an equal weight of liver tissue from the mother. The reality of the passage of alcohol from the mother to the fetus demonstrates the possibility of the intoxication of the fetus.

There is an abundance of data bearing on the effects of parental poisoning on the human offspring, yet almost all of it is complicated. The question arises whether the defects of the offspring are actually due directly to the parental poisoning or to the often degenerate condition of the parent. With lower mammals this question may be controlled, since vigorous individuals with no physical weaknesses may be selected for study. One of the most interesting human cases is that Forel cites as recorded by Schweighofer. A normal woman married a normal man and had three sound children. The husband died and the woman married a drunkard and gave birth to three other children; one of these became a drunkard; one had infantilism, while the third was a social degenerate and drunkard. The first two of these children contracted tuberculosis, which had never before been in the family. The woman married a third time and by this sober husband again produced sound children. This is a logical experiment, the female was first tested with a normal male and gave normal children; when mated with an alcoholic male the progeny were defective. She was later tested again with a normal male and found to be capable of producing sound offspring. A number of such cases are on record but all are open to the question whether the defective offspring are actually due to the effects of the poison on the parent, or to the fact that the parent may have been weak and degenerate from the beginning.

Other substances than alcohol seem to act directly on the germ cells of man and mammals, and these actions are more important since there is no reason to believe, for some of them at any rate, that they accompany a degenerate condition. Constantine Paul long ago pointed out that the children of lead workers were often defective.

He made the interesting observation that when the father alone was employed in such work his children were affected. In 32 conceptions with such fathers 12 resulted in premature labor and stillbirths, 20 living births occurred but only 3 children survived. Eight died the first year, 4 the second, and 5 the third year.

Mairet and Cambemale in 1888 were the first to experiment on the influence of alcohol on the mammalian offspring. They treated a dog for 8 months with absinthe (11 gr. per day per kilo of animal weight) and paired this alcoholized dog with a normal bitch. Twelve young resulted; 2 were born dead, 3 died within 14 days, and the others died between 32 and 67 days of intestinal catarrh, tuberculosis, etc. In a second experiment, both parents were mated while normal, then the female was treated for 23 days (2.75 to 5 gr. of absinthe of 72 per cent. per day per kilo). Of 6 young 3 were stillborn, 2 had normal bodies though of weak intelligence, while one was very sluggish. The evident criticism against this experiment is that an insufficient number of animals was used and there was no control. It is very difficult to rear pups in a laboratory; when apparently perfectly normal, they often die shortly after birth.

Hodge, in 1897, obtained similar results. From one pair of alcoholic dogs he observed 23 pups, 8 were deformed, 9 were born dead, while only 4 lived. In a control set, 41 individuals lived, 4 were deformed, but there were no stillbirths.

Nice has recently published results of treating mice with alcohol. He finds little, if any, effect of the treatment on the offspring. Considering his method of administering the alcohol and the results obtained, the doses used were probably insufficient to produce effects. It may also be possible that mice are more resistant to alcohol than are other mammals. I have discussed these experiments in a previous communication.

EXPERIMENTS

Three years ago a series of experiments were begun on guinea-pigs with the hope of modifying the type of embryo in mammals so as to produce definite monstrosities as one is able to do with lower vertebrates. This primary object has not been fully accomplished, yet the experiments have demonstrated several significant points and have shown that an alcoholized male guinea-pig almost invariably begets a defective offspring even when bred to a vigorous normal female.

Normal, healthy animals are selected for the experiment, and in all cases they are first tested by a normal mating in order to establish their ability to produce vigorous offspring. After such a test the treatments are begun. During the experiments the treated males and females are mated from time to time with normal animals, and in addition, control matings of normal individuals are made. Some of the specimens are treated with alcohol and ether. These substances were used since they readily act upon animal cells and since I had studied their effects on the development of fish embryos and found them to cause rather definite and easily recognizable defects in the central nervous system and organs of special sense.

METHOD AND TECHNIQUE

In the beginning of the experiments alcohol was given along with the food, but the animals ate less and the food did not apparently agree with them. It was then administered in dilute form by a stomach tube; this method disturbed digestion and seemed to upset the animals considerably. It is certain that alcohol given to animals through the stomach deranges their digestion and appetite to such an extent that the experimenter is unable to determine whether the resulting effects are due to the alcohol, as such, or to the general deranged condition of the animal. When given in the drinking water they take little or none of the water and the treatment is insufficient. For these reasons an inhalation method of treatment has been resorted to which, as far as experience goes, has no serious disadvantages and does not complicate the conditions of the experiment.

A fume tank of copper is made of sufficient size to supply breathing space for 4 or 5 guinea-pigs at one time. The tank is arranged with four outlets, so that definite amounts of the fumes may be passed through in a given time and the ventilation controlled. In this way each animal could be given about the same amount of the substances. The individuals, however, differ so in their resistance to the treatment that it has been found better to treat all to about the same degree of intoxication. This physiological index is more reliable as each animal is thus affected in a similar fashion each day. For this purpose they are placed in the fume tank on a wire screen, and absorbent cotton soaked with alcohol is placed beneath the screen, and the animals inhale the fumes. The tank was described and illustrated in a previous article.

Ether is given in a similar manner, except that the animals are much more readily overcome and must be carefully watched while inhaling even the most dilute doses.

In order to avoid handling the females during late pregnancy, a special treating cage is devised. An ordinary box-run with a covered nest in which the animal lives is connected by a drop-door with a metal-lined tank, having a similar screen arrangement to that of the general treatment tank. The pregnant animal may be driven daily into the tank and thus treated with alcohol fumes throughout her pregnancy without being handled in any way that might disturb the developing fetus.

Direct Effects of the Treatment on the Animals

Many of the animals have now been treated almost to the point of intoxication for six days per week for nearly three years. They are affected by the alcohol fumes in different ways; certain ones become drowsy and stupid, while others become excited and sometimes vicious during the treatment, constantly fighting and biting at others in the tank. One male always had to be treated alone on this account. The fumes are inhaled into the lungs and pass directly into the circulation, so that the animals show signs of intoxication very soon after being put into the tank, yet the intake of alcohol is so gradual that they may remain for one hour or more without becoming totally anesthetized. The mucosa of the respiratory tract is considerably irritated during the first few days or weeks of the treatment, but later becomes hardened and little effect can be noticed. The cornea of the eye is greatly irritated and often becomes milky white and opaque during the first few months; but later this clears up in most of the specimens and the animal is able to see perfectly, though one male that has been treated for 32 months is now entirely blind. The general condition of the animals under the treatment is very good; they all continue to grow if treated before reaching their full size, and become fat and vigorous, taking plenty of food and behaving in a normal manner in every particular.

Certain of the animals have been killed at different times during the experiment and their organs and tissues studied microscopically; all have seemed entirely normal. The tissues of one female were examined after she had been treated for over a year, and the heart, stomach, lungs, liver, kidney, etc., were all normal. She was generally fat but there was no fatty accumulation in the parenchyma of any of the organs except possibly a slight excess in the adrenal glands.

As mentioned above several of the animals, both males and females, have been partially castrated during the experiments and the ovaries and testis have been found to be in healthy condition.

The treated animals are, therefore, little changed or injured so far as their behavior and structure goes. Nevertheless, the effects of the treatment are most decidedly indicated by the type of offspring to which they give rise, whether they are mated together or with normal individuals.

EFFECTS ON THE OFFSPRING

The animals have been mated in various combinations. First, treated males have been paired with normal females, the paternal test; this is the crucial test of the influence of the treatment on the germ cells. In this case the chemically modified or weakened spermatozoon can alone be responsible for the defective offspring, since the egg is normal and develops in a normal environment in the healthy mother.

Second, treated females are paired with normal males, the maternal test. This combination offers two chances for injuring the offspring. Either the ovum may be defective as the result of the treatment which the mother has undergone, and may thus give rise to a defective individual; or secondly, the developing embryo may be affected directly by the alcohol in the system of the mother, since Nicloux has shown that this substance may pass from the blood of the mother into the tissues of the fetus. Thus the intoxication of the embryo may modify its forming structures in the same way that a fish embryo develops deformed organs and parts when in sea water to which alcohol has been added.

The third combination is the mating of two alcoholic individuals. This is the most severe test and offers the greatest chance for defective offspring.

Before the experiment or treatment begins the guinea-pigs are all tested by normal matings and are found to give normal vigorous offspring. They continue to give normal offspring until the treatment has lasted for some time. The effect accumulates slowly and is not noticed at once. A number of experiments in which the treatment of a female was commenced at the beginning of pregnancy have so far given rather indefinite results, although a slight effect may be indicated.

In all 124 matings of treated individuals have been made. One hundred and three of these have reached full-term and are recorded. Twenty-one matings are not yet due. From the 103 full-term matings only 52 young have survived and most of these are somewhat under size and show their affected condition in the type of offspring to which they give rise. Yet their parents were all unusually large and originally strong animals.

From 35 control matings 56 healthy offspring have been derived which continue to produce normal animals in the following generations, in a few cases now to the fourth generation.

A tabulated summary of the results may be arranged as indicated in Table 1. The conditions of the animals in the mating pairs are shown in the first column of the table and the total results of the matings are indicated in the following columns.

The first horizontal line gives the record when alcoholic males are paired with normal females. Fifty-nine such matings have reached term, 25 of these gave negative results or early abortions. Some embryos were aborted during very early stages and were generally in such poor condition when found in the cages that little could be learned from them. They were partially or completely eaten by the mother in most cases. The males were always kept with the females during favorable periods for a number of days, usually about three weeks, and conception should have occurred in all cases, as it did with the control matings.

Thirty-four of the 59 matings resulted in conceptions which ran the full term. Eight, or about 24 per cent. of these, were stillborn litters, consisting in all of 15 individuals. Most of these were somewhat premature; in a few cases their eyelids were still closed and the hair was sparse on their bodies. (A normal guinea-pig at birth is well covered with a hairy coat, its eyelids are open and it very quickly begins to run about.)

Twenty-six, or only 44 per cent., of the matings produced litters of living young. These litters contained in all 54 individuals. Twenty-one, or almost 40 per cent., of these young guinea-pigs died within a few days or less than four weeks after birth, while 33 of them survived. *Thus, out of 69 full term young, of which 54 were born alive, only 33 have survived, and many of these are small and excitable animals, and although not treated themselves have since given rise to defective offspring in several cases where they have been mated with one another.* On the other hand, 35 control matings have produced 32 living litters consisting of 60 individuals, only 4 of which have died and 56 are perfectly normal animals.

It is of interest that the young animals before dying show various nervous disturbances, having epileptic-like seizures, and in most cases die in a state of convulsion.

The important fact in the above case is that the father only was alcoholic, the mother being a normally vigorous animal. *This experiment clearly demonstrates that the paternal germ cells may be modified by chemical treatment to such a degree that the treated male will beget abnormal offspring even though he be mated with a vigorous female.* A reconsideration of the figures in the first line of the table shows really how decidedly the injured spermatozoon expresses itself in the fate of the egg with which it combines.

For comparison the second line of the table shows the results of matings between alcoholized females and normal males. These matings might be expected to give more marked results than the previous ones, since in the treated female not only the germ cells may be affected, but the developing embryo itself may be injured by the presence of alcohol in the blood of the mother.

There are 15 matings between alcoholized females and normal males. Three of these, or 20 per cent., gave negative results, or were possibly aborted very early. Three stillborn litters were produced consisting of nine individuals, while 60 per cent. of the matings gave living litters. This result is better by 16 per cent. than that obtained when alcoholized males were paired with normal females. The proportion of surviving individuals is, however, less from the treated females than from the treated males. The 9 living litters contained 19 young, 9 of which died soon after birth and 10 survived. Thus out of 28 full term young only 10, or about 36 per cent., survived, while 64 per cent. of the offspring were lost; in the above cases where the male alone was alcoholic almost 48 per cent. of the full term young survived.

TABLE I

CONDITION OF THE OFFSPRING FROM GUINEA-PIGS TREATED WITH ALCOHOL

Condition of the Animals	Number of Matings	Negative Result or Early Abortion	Stillborn Litters	Number Stillborn Young	Living Litters	Young Dying Soon After Birth	Surviving Young
Alcoholic ♂ by normal ♀	59	25	8	15	26	21	33
Normal ♂ by alcoholic ♀	15	3	3	9	9	9	10
Alcoholic ♂ by alcoholic ♀	29	15	3	6	11	7	9
SUMMARY	103	43	14	30	46	37	52
Normal ♂ by normal ♀	35	2	1	4	32	4	56
2d generation by normal	3	0	0	0	3	0	4
2d generation by alcoholic	3	0	2	5 1 def.	1	0	2
2d generation by 2d generation	19	7	0	0	12	6 1 def.	13
Female treated during pregnancy	4	0	0	0	4	1	7

The third horizontal line indicates the results of pairing alcoholized males with alcoholized females. The effects of the treatment in this case are slightly more marked than in either of the above lines. Twenty-nine such matings gave in 15, or more than 50 per cent., of the cases negative results or early abortions. Three stillborn litters occurred, each consisting of two individuals. Only 11 living litters were produced. These contained 16 young, 9 of which survived while 7 died soon after birth.

A comparison of this combination with the control matings given in the fifth line shows in a decisive manner the really detrimental effects of the treatment. In the one case only 9 surviving young were obtained from 29 matings, while in the other the control animals gave 56 surviving young from 35 matings.

The fourth line summarizes the results of the matings made with treated individuals. A total of 103 matings have run the full term; 43, or almost 42 per cent. of these, have given negative results or early abortions; while 35 control matings failed in only two cases to yield a full term litter. Fourteen, or 13½ per cent., of the matings gave stillborn litters consisting of 30 individuals. Only one stillborn litter occurred in the 35 control matings; this was a large litter of 4 young and the mother seemed almost unable to carry them. The 103 matings of treated animals gave only 46 living litters, about 45 per cent., while 32 living litters, or 91½ per cent., were produced by the 35 control matings. The 46 living litters from the alcoholic individuals contained 89 young, 37 of which died shortly after birth and 52 survived. The 32 living litters from the normal animals consisted of 60 individuals, only 4 of which died while 56, or 93 per cent., of these survived.

Of 119 full term young, 30 of which were stillborn, produced by the alcoholic animals, only 52, or less than 44 per cent., survived as against the 56, or 87½ per cent., survivors among the 64 full-term control offspring.

The bottom line of the table shows that 4 normally mated females treated with alcohol during the period of gestation gave 4 living litters, consisting each of 2 young. One out of the 8 young died soon after birth. These few cases would seem to indicate that the treatment, when started at the beginning of gestation, was not particularly injurious to the embryos developing *in utero*.

Are the Effects on the Offspring Transmitted?

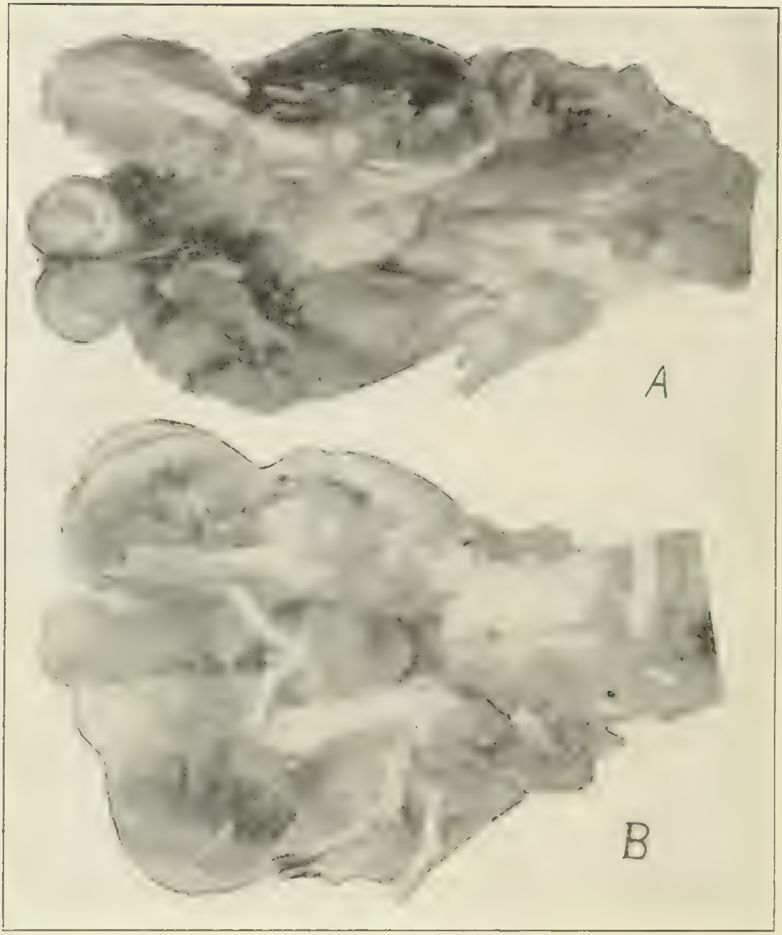
The offspring derived from the alcoholic individuals are termed second generation animals, and were not treated with alcohol. The sixth, seventh and eighth lines of the table represent the data obtained from the few full time matings that have been made with these guinea-pigs.

In three cases second generation animals have been mated with normal individuals and have produced perfect results, though the litters have been small. Three litters are recorded containing a total of 4 young, all of which survived. It might seem as though the normal mate counteracted any defect which may have been present in the second generation animals.

The mating of second generation individuals with alcoholized guinea-pigs gave decidedly different results. The seventh line shows that 2 out of 3 such matings produced stillborn young. In one of these cases the female was of the second generation and the male alcoholic, and in the other case the reverse condition existed; yet both combinations gave dead offspring, one litter of two and one of three individuals. One of these specimens from the second generation female by the alcoholic male was grossly deformed. The third mating gave two surviving young. At present there are too few matings of this combination from which to draw conclusions, yet the results obtained are the most disastrous of all.

Nineteen matings between second generation animals have been made. The outcome in these cases compares very unfavorably with that from the control matings, while the data are much of the same type as those obtained from the alcoholic combinations. Seven, or almost 37 per cent., of the matings gave negative results. Twelve living litters were born consisting of 19 individuals, 6, or about 32 per cent., of which died very soon after birth and showed various nervous disorders. One was entirely eyeless and decidedly deformed.

From the number of records available one might conclude that the effects of the alcoholic treatment were as pronounced upon the offspring of the second generation animals, although they had not been directly treated, as they were upon the offspring of alcoholized individuals. The poison acts upon the cells and tissues of the body, the germ cells as well as other cells, and an offspring derived from the weakened or affected germ cell has all



EXPLANATION OF PLATE

Ventral surfaces of two guinea-pig brains. *A*, the brain of an animal which was entirely eyeless, no optic nerves or tracts are present. *B*, the brain from another member of the same litter. This animal was defective and died shortly after birth, but possessed normal eyes as the photograph shows and the optic nerves are clearly seen passing medially to form the chiasma and from there the optic tracts are shown along the anterior margins of the tuber cineria. All of these optic structures are missing in the brain above.

the cells of its body, both soma and germ, defective since each of the cells is a descendant of the injured germ cell combination.

These are the initial experiments with mammals to show *that an injury of the germ cells may express its effects on the offspring and be passed through subsequent generations.*

The actual outcome of the experiments may be more fully recognized by a consideration of one of the most striking cases. A large normal female, weighing about 700 grams, had given two normal young by a control mating and had since given non-viable young by a mating with an alcoholic male. She was then mated with another large strong alcoholic male which weighed 740 grams and which had given before this mating apparently healthy offspring by normal females. The mating resulted in the production of 4 young, all small and rather excitable in their behavior. These individuals from the normal mother and the alcoholized father grew slowly, although they ate freely and appeared to be well. They remained small and below the average in weight. Three were males and one was a female.

One of the males was mated with a normal female and two normal young resulted. He was then mated with a female from an alcoholic father and she gave birth to two small young; one of these offspring was only half size and very excitable. He was then mated with a female from an alcoholic mother and one small young was produced.

A second one of the three males was mated with a normal female which produced one large apparently normal offspring. He was then mated with a female from an alcoholic father and two small young resulted, one of which died within five days and the other is weak and nervous. He was again mated with a normal female and one normal young was produced.

The third male was mated with his sister and she gave birth to 3 young. One of the young died when one day old, having been in a constant tremor since its birth; another lived for nine days but whenever it attempted to walk it was seized with spasmodic contractions; the third specimen exhibited the same nervous manifestations and was completely eyeless. This animal died eight days after birth and an examination of the brain showed an entire absence of optic tracts, as may be seen in Plate 1*A*.

In the development of this animal it is probable that the optic vesicles were suppressed and never arose from the brain. Thus, no eyes, optic nerves, or optic tracts could have formed. This particular eyeless condition in these experiments is of interest since one is readily able to suppress the origin of the optic vesicles in fish and chick embryos by similarly weakening the embryo with treatments of alcohol, ether, etc.

The mother of these offspring was remated with her brother, but she died six weeks later, not becoming pregnant. She was in an emaciated condition but had always been less than half normal weight.

The three extremely weak and defective offspring were doubtless due to the fact that both of their parents had similarly weakened or injured constitutions, having resulted from a single mating of a normal female with an alcoholized male. The eyeless offspring and the other two nervous non-viable individuals should not be interpreted as due merely to the fact that their parents were brother and sister. Several normal matings of brother and sister have been made during the experiment and perfectly healthy offspring have been produced. In the studies of heredity conducted on guinea-pigs brother and sister are crossed with impunity, in no way weakening their offspring. The significant point in the present consideration is that the two animals coming from the same mother and treated father may have had similar weaknesses or defects and the combination of two such individuals resulted in offspring which exhibited these defects to a more decided extent. The three animals were far more defective than their parents and owed their defects to the modified condition of the germ cells of the grandfather from which they descended.

CONSIDERATION OF INDIVIDUAL MATINGS AND RESPONSES

Studying the data of individual animals, another point of some importance presents itself. This is the fact that the same male often yields very different results when bred to different females, although the females are similar so far as the experiment goes, being either all normal or alcoholic. This may be due to the varying degrees of resistance or hardiness possessed by the germ cells of different individuals. A part of this difference may be due to the fact that as the treatment advances the germ cells are more affected and the results do actually become more pronounced.

Male No. 5 that has been treated with alcohol for two and one half years, has been mated 25 times. Eleven of these matings have yielded negative results; 4 were with alcoholic females and 7 were with normal. Three of the matings gave stillbirths, 2 with normal females and one by an alcoholic female. Only 11 of the 25 matings gave living litters, 4 of these were non-viable and 7 litters survived. Five of the 7 surviving litters were from normal females and 2 from alcoholic mothers. *This alcoholic male has, therefore, begotten only 7 surviving litters out of the 14 full term litters born and out of a total of 25 matings.*

Alcoholic male No. 6 shows a still worse record. Out of a total of 21 matings, 10 have given negative results, 5 by normal and 5 by alcoholic females. Two matings gave stillborn litters, one from a normal female and one from a second generation female. Nine living litters resulted from the 21 matings, 3 from alcoholic females and 6 from normal. Five of the nine litters born alive were non-viable, the young dying soon after birth. *Only 4 litters survived out of the 11 reaching term and out of the total of 21 matings.* Three of these surviving litters were from normal females and one was from an alcoholic mother.

Alcoholic male No. 43 has been mated 10 times. Three of the matings gave negative results, two by alcoholic females and one by a normal. One stillborn litter resulted from a mating with an alcoholic female. Six living litters came from the 10 matings. The young in 3 of these litters died soon after birth and 3 litters survived.

Alcoholic male No. 45 has now been treated about one and one half years and has been mated 9 times. One mating with a normal female gave a negative result. One mating with a normal female gave a stillborn litter. Seven living litters were produced, 5 of which survived, 2 from alcoholic females and 3 from normal females. The data in this case appear slightly better than in the foregoing but this is due to the fact that only a few matings have been made and most of these during the early stages of the treatment, when the effects are not so pronounced.

All of the other alcoholized males in the experiments show comparable records.

A reference to Table I shows that with three exceptions 35 matings of normal males with normal females gave living litters containing in all 60 individuals, only 4 of which failed to survive. This record stands in striking contrast to the data recorded above from the 4 alcoholic males, and it shows convincingly that the alcoholic treatment has affected the germ cells of these males so that they are no longer capable of producing entirely normal offspring even though they be mated with normal females.

The outcome of the successive matings of fifteen different females is tabulated in Table II. The varying ways in which the same individual has responded in different matings is noticeable. Number 15, a normal female, shows an instructive record. Mated with alcoholic male No. 6 she gave two stillborn young; mated with alcoholic male No. 5 a negative result; remated with No. 5, two young were born and both died of convulsions during the fourth week; then mated with a normal male a normal vigorous offspring was produced; mated again with alcoholic male No. 5 an apparently normal guinea pig was born; mated with No. 43, an alcoholic male, she gave 2 young which only lived for two days; then mated again with a normal male she produced 2 vigorous offspring and finally mated with No. 69, an etherized male, 2 young were born, one of which died at birth. Thus, out of the 8 matings, 2 with normal males gave perfectly normal offspring, while 5 out of the 6 matings with treated males gave disastrous results and only one of these matings resulted in the production of an apparently normal young.

Number 56, a normal female, mated to a normal male, No. 48, gave 2 normal young; with alcoholic male No. 45 gave 3 premature stillborn fetuses; again to a normal male No. 80 gave 3 normal offspring; and finally again to alcoholic male No. 45 she gave one very small young.

Normal female No. 63 gave two normal individuals by a normal mating and then three successive matings with an alcoholic male failed to produce a viable offspring; one mating resulted negatively, one gave three young dying shortly after birth, and in the third case two late fetuses were aborted. She was then mated again to a normal male and produced two vigorous offspring.

Normal female No. 50 was mated alternately with normal and alcoholic males for 4 matings, with alternately good and bad results.

Animals 19, 30, 54 and 58, all normal females, show records closely similar to the ones just mentioned. (In a former table of successive matings the first mating of No. 19 has been recorded incorrectly; it should read by a normal male giving two normal offspring as in the present Table II, instead of by alcoholic male No. 4 with a negative result.)

Alcoholic female No. 64 gave two normal young by a normal male before her treatment was commenced. Mated to alcoholic male No. 6 she gave two young; one died at birth and one survived; with alcoholic male No. 5 she gave an apparently normal young; finally, by alco-

TABLE II

RESULTS OF THE SUCCESSIVE MATINGS OF FIFTEEN FEMALES

Animal	1st Mating	2d Mating	3d Mating	4th Mating	5th Mating	6th Mating	7th Mating	8th Mating
No. 64 alc.	Nor. ♂, 2 nor. young	Alc. ♂ 6, 2 young, 1 died	Alc. ♂ 5, 1 nor. young	Alc. ♂ 43, 2 foetuses, died *in utero*, killed mother	....	....	....	....
No. 59 alc.	Nor. ♂, 3 nor. young	Nor. ♂ 44, 2 young, 1 died	Alc. ♂ 43, 0	Alc. ♂ 43, 0	Nor. ♂ 69, 3 yg., died *in ut.*, killed mother	....	....	....
No. 30 nor.	Nor. ♂ 25, 2 nor. young	Alc. ♂ 45, 2 nor. young	Alc. ♂ 5, 1 nor. young	Alc. ♂ 5, 2 foetuses, died *in utero*, killed mother	....	....	....	....
No. 15 nor.	Alc. ♂ 6, 2 still-born	Alc. ♂ 5, 0	Alc. ♂ 5, 2 young, both died in convulsions	Nor. ♂, 1 nor. young	Alc. ♂ 5, 1 apparently normal	Alc. ♂ 43, 2 young, both died 2d day	Nor. ♂ 70, 2 nor. young	Ether ♂ 69, 2 young, 1 died
No. 19 nor.	Nor. ♂, 2 nor. young	Alc. ♂ 6, 1 still-born	Alc. ♂ 6, 0	Alc. ♂ 5, 4 small, ½ size young	Alc. ♂ 45, 2 young, 1 died	Alc. ♂ 6, 0	Alc. ♂ 6, 3 young, 2 died	
No. 63 nor.	Nor. ♂ 44, 2 nor. young	Alc. ♂ 5, 0	Alc. ♂ 5, 3 young, all died	Alc. ♂ 5, 2 late foetuses aborted	Nor. ♂ 93, 2 nor. young	....	....	....
No. 50 nor.	Nor. ♂ 46, 3 nor. young	Alc. ♂ 43, 2 young, both died	Nor. ♂ 69, 2 nor. young	Alc. ♀ 43, 0	....	....	....	....
No. 54 nor.	Nor. ♂ 44, 2 nor. young	Alc. ♂ 6, 2 young, both died	Nor. ♂ 72, 2 nor. young	Alc. ♂ 43, 1 young	....	....	....	....

Animal	1st Mating	2d Mating	3d Mating	4th Mating	5th Mating	6th Mating	7th Mating	8th Mating
No. 55 alc.	Nor. ♂, 2 nor. young	Alc. ♂ 6, 0	Nor. ♂ 47, 2 young, both died	Alc. ♂ 6, 1 young	....	....	....	....
No. 56 nor.	Nor. ♂ 48, 2 nor. young	Alc. ♂ 45, 3 premature, stillborn	Nor. ♂ 80, 3 nor. young	Alc. ♂ 45, 1 young, very small	....	....	....	....
No. 58 nor.	Nor. ♂ 46, 2 nor. young	Alc. ♂ 6, 2 nor. young	Alc. ♂ 43, 1 young, died at birth	Alc. ♂ 6, 0	....	....	....	....
No. 60 alc.	Nor. ♂ 46, 3 young, all died	Nor. ♂ 44, 2 nor. young	Alc. ♂ 45, 1 stillborn	Alc. ♂ 43	....	....	....	....
No. 62 alc.	Nor. ♂ 47, 2 nor. young	Nor. ♂ 46, 2 small young	Alc. ♂ 6, 2 young, died 2d day	Alc. ♂ 45, 2 young, died 2d day	....	....	....	....
No. 65 nor.	Nor. ♂, 2 nor. young	Alc. ♂ 5, 2 small young	Alc. ♂ 6, 2 small young	Nor. ♂ 83, 2 young, 1 died, ♀ treated during pregnancy	....	....	....	....
No. 66 alc.	Nor. ♂, 1 nor. young	Nor. ♂ 44, 2 nor. young	Alc. ♂ 5, 0	2d gen. ♂ 102, 3 fetuses aborted	2d gen. ♂ 102, 2 small young	....	....	....

holic male No. 43 she produced two fetuses which died *in utero* and killed her.

Female No. 59, alcoholic, has a similar record and was also finally killed by 3 fetuses dying *in utero* and poisoning her.

Alcoholic females 55, 60, 62 and 66 all show very poor records in the production of viable offspring.

During the course of the experiments four females have been killed by the death of fetuses *in utero*. In three of the cases this occurred after the females had been treated with alcohol for a number of months and were becoming more and more affected by the treatment. No. 61 died after she had been given alcohol for four months with three fetuses *in utero* which had apparently been dead for several days. No. 64 had been treated with alcohol for over one year and finally, while in a late stage of pregnancy by alcoholic male No. 43, she became stupid and refused to eat. On examination the fetuses showed no signs of life and were quite hard; they were removed by operation and had been dead several days. The mother had become so intoxicated by this condition that she was unable to recover after the removal of the fetuses.

Female No. 59 had been treated with alcohol for thirteen months when, after being mated with a normal male, she was operated upon in order to remove three dead fetuses. She failed to recover.

The fourth case of young dying *in utero* was that of a normal female, No. 30, that had been mated with an alcoholic male. The almost full term fetuses died and produced the same symptoms in the mother as those in the cases above; she was also operated upon and failed to recover.

It is a perfectly easy operation to remove the ovaries or uterus from a normal guinea-pig. I have not tried to remove living fetuses. There is little doubt, however, that it is the accumulated toxins owing to the presence of the dead fetuses which prevented the females from recovering after the operation in these three cases.

The death of the late fetuses *in utero* is to be expected merely as a step in the series. A number of early abortions of embryos have occurred, and the table shows the enormous fatality among the young shortly after birth, as well as the frequent occurrence of stillborn litters. When the young happen to die shortly before birth instead of after birth, the female in some cases is unable to expel them from the uterus.

A consideration of a few of the notes made from the individual matings will further serve to illustrate the actual response of the animals to the treatment as shown by the outcome of the matings. For this purpose we may take two random groups.

First a group of eleven matings made on October 30 and 31, 1912, resulted as shown in the following notes:

Oct. 30, Nor. ♀ No. 30 × Alc. ♂ 5 = Jan. 9, 1913—One normal young, No. 123 ♀.

Oct. 30, Nor. ♀ No. 29 × Alc. ♂ 45 = Jan. 20, 1913—Three very small young, Nos. 134 ♂, 135 ♀, and 136 ♀.

Oct. 30, Nor. ♀ No. 58 × Alc. ♂ 6 = Jan. 15, 1913—Two normal young, Nos. 129 ♂, 130 ♀.

Oct. 30, Alc. ♀ No. 62 × Nor. ♂ 46 = Jan. 9, 1913—Two small but normal young, Nos. 121 ♀, 122 ♀.

Oct. 30, Alc. ♀ No. 59 × Alc. ♂ 43 = 0.—*Only one of the eleven matings that did not take.*

Oct. 30, Alc. ♀ No. 60 × Nor. ♂ 44 = Jan. 17, 1913—Two apparently normal young, Nos. 131 ♂, 132 ♂.

Oct. 31, Nor. ♀ No. 68 × Nor. ♂ 69 = Jan. 21, 1913—Three normal young, Nos. 137 ♂, 138 ♂, 139 ♂.

Oct. 31, Nor. ♀ No. 71 × Nor. ♂ 72 = Jan. 10, 1913—Two small normal young, Nos. 126 ♂, 127 ♂.

Oct. 31, Nor. ♀ No. 73 × Nor. ♂ 70 = Jan. 19, 1913—One large young, No. 133 ♀.

Oct. 31, Nor. ♀ No. 74 × Nor. ♂ 79 = Jan. 11, 1913—One large young, No. 128 ♀.

Oct. 31, Alc. ♀ No. 55 × Nor. ♂ 47 = Jan. 9, 1913—Two small and weak young. Nos. 124 ♀, 125 ♂, both died when one month old.

In the group of 11 matings, 4 were between normal males and normal females, control matings, three were between alcoholic males and normal females, and three between normal males and alcoholic females. All of these produced offspring as noted, while the single mating between an alcoholic male and an alcoholic female gave a negative result although the animals were kept together as long as the individuals of any of the other pairs, 17 days.

One of the alcoholic females gave two young which died within a month.

A second group of 18 matings made during November, 1912, are recorded as follows:

Nov. 4, 2d Gen. ♀ 100 × 2d Gen. ♂ 92 = 0.—Together very long, though failed to take.

Nov. 4, 2d Gen. ♀ 101 × 2d Gen. ♂ 99 = 0.—Together very long, though failed to take.

Nov. 15, 2d Gen. ♀ 91 × 2d Gen. ♂ 92 = Feb. 18, 1913—One large young, apparently normal, No. 170 ♂.

Nov. 15, 2d Gen. ♀ 98 × 2d Gen. ♂ 99 = 0.—Failed to take, though together six weeks.

Nov. 16, 2d Gen. ♀ 76 × 2d Gen. ♂ 77 = Jan. 28, 1913—Three defective young, one eyeless, all died within 10 days.

Nov. 16, Nor. ♀ 88 × 2d Gen. ♂ 78 = Feb. 20, 1913—One large young apparently normal, No. 171 ♂ (long gestation).

Nov. 16, Alc. ♀ 66 × Alc. ♂ 5 = 0.

Nov. 16, Nor. ♀ 87 × 2d Gen. ♂ 75 = Jan. 27, 1913—Two normal young, Nos. 140 ♀, 141 ♂.

Nov. 16, Nor. ♀ 19 × Alc. ♂ 6 = Jan. 30, 1913—Three small weak young, two died shortly after birth, one No. 146 ♂ survived.

Nov. 16, Nor. ♀ 34 × Alc. ♂ 43 = Feb. 4, 1913—Two normal young, Nos. 156 ♀, 157 ♂.

Nov. 16, Nor. ♀ 49 × Alc. ♂ 45 = Jan. 31, 1913—Three normal young, Nos. 147 ♂, 148 ♀, 149 ♀.

Nov. 16, Nor. ♀ 33 × Nor. ♂ 46 = Jan. 30, 1913—Two fine young, Nos. 144 ♂, 145 ♀.

Nov. 23, Nor. ♀ 50 × Nor. ♂ 69 = Feb. 5, 1913—Two large young, Nos. 160 ♂, 161 ♀.

Nov. 23, Nor. ♀ 15 × Nor. ♂ 70 = Feb. 4, 1913—Two large young, Nos. 158 ♀, 159 ♂.

Nov. 23, Nor. ♀ 54 × Nor. ♂ 72 = Feb. 3, 1913—Two large young, Nos. 150 ♂, 151 ♂.

Nov. 23, Nor. ♀ 56 × Nor. ♂ 80 = Feb. 3, 1913—Three normal young, Nos. 152 ♂, 153 ♂, 154 ♀.

Nov. 23, Nor. ♀ 52 × Nor. ♂ 81 = Feb. 12, 1913—One large young, 167 ♀.

Nov. 23, Nor. ♀ 53 × Nor. ♂ 48 = Feb. 3, 1913—One large young, 155 ♂.

To summarize these 18 pairings: 7 were normal control matings all giving vigorous young. Three were alcoholic males by normal females, all gave young. Two litters consisted of small animals, while the third litter was very weak, two of its members died just after birth.

Five of the matings were made between untreated animals which came from alcoholic parents, second generation animals. One litter of 3 individuals was born, all were weak and defective, one being eyeless and the 3 died within ten days. One other litter consisted of only one individual which was born after an unusually long period of gestation. Three of the matings gave negative results. Thus 3 out of 5 second generation matings failed to take, one gave non-viable young, and only one litter of viable young was produced from the 5 matings, as against 7 viable litters from the 7 control matings.

There were two matings of second generation males by normal females. Both of these gave viable young, though one of the females had an unusually long gestation period. The normal mates seemed to have counteracted the weakened condition of the second generation males.

The one mating between two alcoholic individuals again gave negative results.

Thus 4 of the 18 matings failed to take. 3 of these were between second generation animals and the fourth was the double alcoholic mating.

Fourteen matings were successful, in five cases one member of the pair was normal and in seven cases both were normal. In the remaining two cases both animals were of the "second generation," though themselves untreated, one litter was non-viable and but a single litter of one young survived.

These sample notes from 29 pairs out of the total of 167 full term matings contained in Table I, gives a fairly clear idea of the manner in which the individual animals respond.

Conclusions

Finally, in conclusion, we may consider the type or nature of the injury produced by the treatments and the manner of transmission or inheritance involved. The treated animals themselves show no effects of nervous or systemic injuries in their general health or behavior. It is only when such individuals are bred that they prove to be inferior to the untreated animals. This inferiority is shown both by a slowness or failure in many cases to conceive, although they copulate normally, and by the poor quality of the offspring to which the successful conceptions give rise. That this poor quality of offspring is due to an injury inflicted by the treatment on the germ cells of the alcoholic animals is shown by the fact that when the male alone is treated the offspring he begets are decidedly inferior. The germinal taint is still further demonstrated by the fact that the offspring from treated parents although themselves not treated produce equally or more defective young than do the treated animals.

The defects shown by the offspring of alcoholic parentage are general in type, not definite or specific. The central nervous system and special sense organs are apparently most affected, and this is true also in embryos developing in unfavorable environments. I have found that fish embryos when developed in a large number of unusual environments, including alcohol and ether, always show marked abnormalities of the nervous system and special sense organs, particularly of the eyes and ears. When chick embryos are subjected to similar environmental conditions, it has been found in experiments performed during the last two winters, that they respond in a manner similar to the fish. Many chick embryos show different degrees of cyclopia and the degeneration or absence of one eye of the normal pair is a common defect in the chick as it is in the fish where many grades of monophthalmicum asymmetricum were described in my communications on the subject. In this connection the eyeless guinea-pig derived from untreated animals that had an alcoholic father becomes of special interest, and the general nervous symptoms, spasms, epileptic-like seizures, etc., shown by animals of two generations gain importance.

All defects of the nature of those mentioned may be considered as due to weakened development or developmental arrest. Any environment which weakens or retards the early stages of development will cause such conditions. How, then, are they transmitted by the alcoholic male, or by the untreated offspring of alcoholic parentage?

When the animal is treated with alcohol, lead or almost any poison for a long period of time, the poison acts to weaken or injure all of the body tissues with which it comes in contact through the circulation, the liver and other glandular organs usually show the effects in particular. The reproductive glands are injured as well as others and all the cells and tissues of such an organ are below normal. When such a male animal is paired with a normal female, the resulting offspring contains in every cell of its body elements derived from the weak or injured male pronucleus. Unless the vigor of the normal parent is sufficient to overcome the injured condition, the offspring is defective.

The important thing in considering this defective offspring is the recognition of the fact that not only its soma cells but its germ cells as well are defective, since all were derived from the modified spermatozoon of the injured father. When this offspring with injured germ cells is paired with a similar individual, as has been frequently done in the experiments described, the resulting animal body is constituted of cells, all of which are the result of proliferation or division from the primary injured egg and sperm cell; thus all of the cells are of a similar inferior nature. Therefore, the young derived from the second generation should be, leaving out of consideration the power of a cell to recover from such poisoning, equally as defective as those derived from the treated parents.

This might be construed to show the transmission of acquired characters, but it can not be properly interpreted in such a sense. There is in this case no transmission of new or strange characters strictly speaking, merely a weakened or injured cell gives rise to other weak cells. The term "weak" is employed for the lack of a better one, meaning that the cells are below normal in reaction, respond slowly or in a deranged manner and often die or wear out early in their career.

It may be that in nature such defects as hare-lip and cleft palate are transmitted in a fashion similar to the method just suggested. These defects run in families and are said to be inherited. Their character, however, is clearly that of a developmental arrest. Such defects are very probably not truly inherited at all, that is, they are not definite characters or qualities as hair and eye color are, but are due to the fact that the germ cells from which the deformed individual arose, or the uterine environment in which it developed, were not fully normal in vigor. A more careful study of the inheritance of such defects will doubtless reveal the fact that other deformities and developmental arrests are also common in the same families. In other words, weak germ cells or the poor developmental environment runs in the family, and hare-lip and cleft palate are merely the external expressions of these conditions.

The interpretation may be concretely expressed as follows: Mammals treated with injurious substances, such as alcohol, ether, lead, etc., suffer from the treatments by having the tissues of their bodies injured. When the reproductive glands and germ cells become injured in this way they give rise to offspring showing weak and degenerate conditions of a general nature, and every cell of these offspring having been derived from the injured egg or sperm cell are necessarily similarly injured and can only give rise to other injured cells and thus the next generation of offspring are equally weak and injured, and so on. The only hope for such a line of individuals is that it can be crossed by normal stock, in which case the vigor of the normal germ cell in the combination may counteract, or at any rate reduce, the extent of injury in the body cells of the resulting animal. By continually introducing normal mates into such a line the defects might be entirely eliminated, but the continued interbreeding of animals with defects or systemic injuries will doubtless result in the death of the race.

The offspring from a diseased father derives all of its cells from the poor sperm, thus each cell is poor in part and is so passed from generation to generation.

The present experiments are being continued and a large number of matings between second and third generation animals are now made. Various combinations of second generation animals are being tried in order to compare the effects resulting from paternal and maternal treatments, as well as the doubled effects. Two animals, both derived from alcoholic fathers, are mated, others from alcoholic mothers, and the various crosses between these classes are tried. In other cases second generation sisters are mated one with a normal and the other with an alcoholic male, and subsequently these matings will be reversed in order to study the power of the normal mate to counteract the injured condition, as well as the tendency of new alcoholic cells to augment the condition.

SUMMARY

Three years ago a series of experiments were begun with guinea-pigs in order to test the possibility of modifying the type of development in mammals, so as to produce definite monstrosities, as had been accomplished with lower vertebrates. This primary object has not been fully attained at the present time, yet the experiments have demonstrated several points concerning injury of the germ cells, and have shown that an alcoholized male guinea-pig almost invariably begets defective offspring even when mated with a vigorous normal female.

A method has been devised for administering the alcohol by inhalation. The animals inhale the fumes of 95 per cent. alcohol which are readily taken into the pulmonary circulation, and very soon cause a state of intoxication. By this method the stomach is not injured and the general metabolism of the animal is maintained in a healthy condition. Few changes are produced in the tissues of the animals, even after a treatment given six times per week has extended over almost three years. Yet the actual effects upon the reproductive glands are indicated by the inferior quality of the offspring to which the alcoholized individuals give rise.

The animals have been mated in various combinations. First, alcoholized males are paired with normal females, the paternal test, and also the crucial test of the influence of the treatment on the germ cells. Fifty-nine such matings have reached term. Twenty-five of these gave negative results or early abortions. Thirty-four of the fifty-nine matings resulted in conception which ran the full term. Eight, or about 24 per cent., of these were stillborn litters containing in all 15 dead individuals. Many of them were somewhat premature. Twenty six, or only 44 per cent., of the matings produced litters of living young, containing a total of 54. Twenty one, or almost 40 per cent., of these young animals died within a few days or less than four weeks after birth and only 33 of them survived. Many of the 33 survivors are small excitable animals and though not treated themselves have usually given rise to defective offspring in the several cases where they have been mated with one another.

The second combination is between alcoholized females and normal males, the results of which are interesting in comparison with the above. In this combination there are two chances to injure the offspring; in the first place it may arise from a defective egg cell, or secondly, it may be injured by an abnormal developmental environment within the body of the alcoholized female. Fifteen such matings have been made. Three of these, or 20 per cent., gave negative results, or were possibly aborted very early. Three stillborn litters of nine individuals were produced. Sixty per cent. of the matings gave living litters, as against 44 per cent. in the first combination between treated males and normal females. The proportion of surviving young is, however, less from the treated females than from the treated males. Of 19 living young, 9 died soon after birth and 10 survived.

The third combination was between alcoholized males and females. Twenty-nine such matings gave in 15, or more than 50 per cent., of the cases negative results or early abortions. Three stillborn litters occurred, each consisting of two individuals. Only 11 living litters were produced containing 16 young, 9 of which survived while 7 died soon after birth.

All of the matings of the treated animals may be combined and compared with control matings as follows: In a total of 103 full term matings, 43, or almost 42 per cent., have given negative results or early abortions, while 35 control matings failed in only two cases, or about 6 per cent., to yield a full term litter. Fourteen, or 13½ per cent., of the matings gave stillborn litters consisting of

30 dead individuals. Only one stillborn litter occurred in the 35 control matings; this was a large litter of 4 individuals and the mother seemed almost unable to carry them. The 103 matings gave only 46 living litters, about 45 per cent., while 32 living litters, or 91½ per cent., were produced by the 35 control matings.

The 46 living litters from the alcoholic matings contained 89 young, 37 of which died shortly after birth and 52 survived. The 32 living litters from the normal animals consisted of 60 individuals only 4 of which died while 56, or 93 per cent., of them survived.

Of 119 full term young, living and stillborn litters, produced by the alcoholic animals only 52, or less than 44 per cent., survived as against the 56, or 87½ per cent., survivors among the 64 full term control offspring.

The offspring derived from the alcoholic individuals are termed second generation animals and were not themselves treated with alcohol. In three cases second generation individuals have been mated with normal and have given perfect results, although the litters have been small. It might seem as though the normal mate possessed a strong tendency to counteract any defect which may have been present in the second generation animal.

Mating second generation individuals with alcoholized guinea-pigs gave very different results. Two out of three such matings produced stillborn young, one of which was grossly deformed. The third mating gave two surviving young.

Nineteen matings have been made between second generation animals, the outcome of which compares very unfavorably with that from the control matings, while the data are closely similar to those obtained from the alcoholic matings. Seven, or almost 37 per cent., of the matings gave negative results. Twelve living litters were born consisting of 19 individuals, 6, or about 32 per cent., of which died very soon after birth and showed various nervous disorders; one was entirely eyeless and decidedly deformed.

From the number of records available one might conclude that the effects of the alcoholic treatment were as pronounced upon the offspring of the second generation animals, although they had not been directly treated, as upon the offspring of alcoholized individuals. The poison injures the cells and tissues of the body, the germ cells as well as other cells, and the offspring derived from the weakened or affected germ cells have all of the cells of their bodies defective, both soma and germ, since each of the cells is a descendant of the injured germ cell combination. In this manner the defects or degenerate conditions are transmitted or passed to subsequent generations.

LITERATURE

Bataillon, E. 1910. Le problème de la fécondation circonscrit par l'imprégnation sans amphimixie et la parthénogénèse traumatique. *Arch. Zool. Exp.*, Tm. 6, Nr. 2.

Bertholet, E. 1909. Ueber Atrophie des Hoden bei chronischem Alkoholismus. *Centrlb. f. allg. Path.*, XX.

Forel, A. 1911. Alkohol und Keimzellen (blastophthorische Entartung). *Münchener med. Wochnschr.*, LVIII.

Herbst, C. 1909. Vererbungsstudien. VI. *Arch. f. Entw'mech.*, Bd. 27.

Hertwig, G. 1912. Das Schicksal des mit Radium bestrahlten Spermachromatins im Seeigelei. *Arch. f. Mikr. Anat.*, Bd. 79, Abt. II.

———. 1913. Parthenogenesis bei Wirbeltieren, hervorgerufen durch artfremden radiumbestrahlten Samen. *Arch. f. Mikr. Anat.*, Bd. 81, Abt. II.

Hertwig, O. 1913. Versuche an Tritoneiern über die Einwirkung bestrahlter Samenfäden auf die tierische Entwicklung. *Arch. f. Mikr. Anat.*, Bd. 82, Abt. II.

Hertwig, P. 1911. Durch Radiumbestrahlung hervorgerufene Veränderungen in den Kernteilungsfiguren der Eier von *Ascaris megalocephala*. *Arch. f. Mikr. Anat.*, Bd. 77, Abt. II.

— —. 1913. Das Verhalten des mit Radium bestrahlten Spermachromatins im Froschei. Ein cytologischer Beweis für die parthenogenetische Entwicklung der Radiumlarven. *Arch. f. Mikr. Anat.*, Bd. 81, Abt. II.

Kyrle. 1909. Bericht über Verhandlungen der XIII Tagung der Deutschen pathologischen Gesellschaft in Leipzig. *Centralb. f. Path. u. path. Anat.*, Bd. XX, Nr. 77.

Loeb, J. 1908. Ueber die Natur der Bastardlarve Zwischen dem Echinodermenei und Molluskensamen. *Arch. f. Entw'mech.*, Bd. 27.

Lustig, A. 1904. Ist die für Gifte erwordene Immunität übertragbar von Eltern auf die Nachkommenschaft? *Centralb. f. Path.*, Bd. XV.

Mairet et Combemale. 1888. Influence dégéneration de l'alcool sur la descendance. *Compt. rend. Acad. Sc.*, CVI.

Nice, L. B. 1912. Comparative Studies on the Effects of Alcohol, Nicotin, Tobacco Smoke, and Caffeine on White Mice, I. Effects on Reproduction and Growth. *Journ. Exp. Zool.*, VII.

Nicloux. 1900. Passage de l'Alcool ingéré de la mère au fœtus, etc. *L'Obstetrique,* Tm. XCIX.

Paul, C. 1860. Etude sur l'intoxication lente par les préparations de plomb, de son influence sur le produit de la conception. *Arch. gén. de Méd.*, Tm. XV.

Simmonds, H. 1898. Ueber die Ursache der Azoospermie. *Berl. Klin. Wochschr.*, Nr. 36.

Stockard, C. R. 1910. The Influence of Alcohol and other Anæsthetics on Embryonic Development. *Am. Journ. Anat.*, X.

———. 1912. An Experimental Study of Racial Degeneration in Mammals Treated with Alcohol. *Arch. Internal Med.*, X.

Stockard, C. R. and Craig, D. M. 1912. An Experimental Study of the Influence of Alcohol on the Germ Cells and the Developing Embryos of Mammals. *Arch. f. Entw'mech.*, Bd. XXXV.

Sullivan, W. C. 1899. A Note on the Influence of Maternal Inebriety on the Offspring. *Journ. Ment. Sci.*, XLV.

Todde, C. 1910. L'azione dell'alcool sullo svillupo e sulla funzione dei testicoli. *Riv. sper. di Freniatria*, Vol. XXXVI, Nr. 3.

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AN EXPERIMENTAL STUDY OF THE POSITION OF THE OPTIC ANLAGE IN AMBLYSTOMA PUNCTATUM, WITH A DISCUSSION OF CERTAIN EYE DEFECTS

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NINE FIGURES

INTRODUCTION

Numerous investigations have recently been directed towards an analysis of the processes concerned in the development of the vertebrate eye. Both mechanical and chemical methods of experiment have been employed and at the present time most of the results seem open to explanation. Various theories and speculations, however, have been advanced which will probably form a source of contention until the experimental results are better or more uniformly interpreted.

By way of introduction to the experiments recorded in the present paper and a discussion of their significance, it may be well to outline briefly the status of the main problems concerned.

The writer has previously recorded the results of experiments bearing upon an analysis of the manner of origin and development of the optic cup and the optic lens. It was demonstrated that in the species studied the crystalline lens can arise entirely independent of any influence from other eye parts. It seems also equally clear from the many cases observed that the optic vesicle or cup can at some stage during its development induce the formation of a lens from the ectoderm with which it comes in contact, even though this ectoderm is not exactly that from which the normal lens would have arisen. Lenses were induced to arise only from head ectoderm and independent lenses invariably arose in the head region, it was thus concluded that the head ectoderm principally possessed the independent lens forming power.

A consideration of numerous eye abnormalities which occurred in these experiments seemed to throw light on the earlier conditions of origin of the optic anlage from the medullary tissues. A number of the abnormal eyes appeared to lend themselves to a common interpretation and for this reason I advanced in an entirely hypothetical manner the view that these conditions might be considered as arrests in eye development.

Spemann in two more recent contributions to the development of the eye has confirmed all of my observations that were touched upon by his experiments. In an equally general manner he has disagreed with most of the deductions which were drawn from my experimental results. An attempt to satisfy these disagreements will be made in the body of the present paper.

It is now concluded from a study of abnormal eyes and the experiments below that the eye anlage in the medullary plate is primarily median and single and normally separates or spreads into two almost equal growth regions which develop in lateral directions reaching further and further out until finally the optic vesicles come in contact with the ectoderm at the sides of the head. Provided this view is correct cyclopia is then an arrest in eye formation.

Spemann, on the contrary, holds that the eye anlagen originally arise lateral in position along the borders of the medullary plate. The cyclopean defect according to him is due to a failure of central medullary tissue to develop so that the lateral eye anlagen slump towards the median plane, *fuse* and form a single cyclopean eye. Spemann, however, presents no experimental evidence to show that the eye anlagen do occupy lateral positions since all of his operations included the median medullary tissue as well as the lateral.

The only evidence to draw upon is the interesting experiments of Lewis, in which it was found that cyclopia sometimes resulted in Fundulus embryos when the anterior end of the embryonic shield was injured by pricking. Lewis also interpreted these defects as due to a fusion of the optic anlagen, and had suggested, as Spemann now does, that the chemicals used in my experiments suppressed the development of median tissue in the medullary plate and thus caused the eye anlagen to come together, fuse and produce cyclopia. There are a great many strong objections to this hypothesis of Lewis and Spemann which have been enumerated in my previous papers and to which I shall take occasion to refer briefly in the following discussion.

An objection of primary importance to the idea of cyclopia as a result of the coming together of lateral anlagen through a failure of intermediate tissue to form is the fact that cyclopean eyes are rarely in size and extent equal to the sum of the two normal eyes combined. A cyclopean eye is, as a rule, little if any larger than one normal lateral eye and in fact is often much reduced or actually minute in size as compared with a normal eye. This fact indicates most decidedly that eye material, as such, has been injured or arrested in its development and differentiation. One is then scarcely warranted in assuming that the defect is solely due to a failure in formation of material between the eyes.

Spemann has found, although he locates the anlagen in the wrong place, that not only is the eye anlage definitely localized in the open medullary plate but actually the tapetum nigrum is distinct from the other retinal layers. How then could absence of material between the *lateral* eye anlagen cause less eye material to arise?

The differentiation of these prelocalized anlagen require definite amounts of energy. Any treatment that weakens the developing embryo at certain periods in a definite way renders the eye anlagen incapable of differentiation so that they do not arise from the brain.

The entire problem is readily open to experimental test. The contention may resolve itself into the question: Where are the optic anlagen originally located in the medullary plate or tube? The present communication will present the results of experiments aimed towards an answer to this question. Certain interpretations put forward by Spemann regarding the origin and development of the primary components of the eye will also be considered.

MATERIAL AND METHOD

The material used in all the experiments has been the developing embryos of the salamander, Amblystoma punctatum. Amblystoma eggs are surrounded by masses of jelly-like material from which they may readily be separated. The eggs live perfectly without the jelly mass, provided they are well covered with fresh water.

These eggs are of sufficient size to render it possible to cut out with fair accuracy definite regions or groups of cells from the medullary plate or groove.

The method of experiment has been entirely by mechanical operation. This method is particularly useful for the problem in hand since definite areas may be removed and the results studied. The operations were made under a binocular microscope. Fine steel needles and the smallest scissors were used as instruments.

The embryos were kept from three to five days after the operation in water which had been previously boiled. They were killed in a mixture of one part formalin to three parts saturated corrosive sublimate, left for three hours, rinsed in water and put into 70 per cent alcohol with iodine. Others were fixed for three hours in Bouin's fluid, formalin and picroacetic. The sectional embryos were stained with Delafield's hematoxylin and eosin.

CONSIDERATION OF THE EXPERIMENTS

1. Sticking the future brain portion of the medullary plate with needles

The anterior portion of the medullary plate was stuck with steel needles in such a manner as to disturb the cells over an area extending from the anterior border of the medullary fold back to the constricted portion of the plate and laterally from fold to fold. The needle was inserted below the outer layer of cells and raised so as to push the cells apart; this was done a number of times with each specimen. The needles were also swung to the right and left in the medullary tissue until the cells were considered to be disturbed to quite an extent. The object in such an experiment was to determine how severe an injury to the cells was necessary in order to prevent the development of the optic vesicles.

Twenty-three embryos were treated in this manner, and all were killed four days after the operation. Under a high power binocular microscope most of them distinctly showed that the optic cups were well pushed out laterally and in contact with the ectoderm at the sides of the head. Both eyes were clearly seen in seventeen of the individuals, while six seemed to have eyes yet not so well developed. These six doubtful specimens were sectioned and studied microscopically.

Both eyes were present and apparently normal in structure in five of the six embryos. The sixth individual showed eyes which were slightly irregular in form and poorly developed, yet both eyes were distinctly present.

The experiment would indicate that a disturbance, of the type employed, of the cells constituting the eye anlage in the medullary plate was not sufficient to prevent the normal development of the eyes in these embryos.

Another point of interest might have been attacked by such an experiment provided the embryos had been allowed to develop sufficiently long after the operation. That is, whether or not the cells destined to form the tapetum nigrum layer might be intermixed with those cells destined to form other retinal layers, and so produce an eye with the pigment cells scattered irregularly through the retina. Spemann's recent experiments on cutting out and reversing certain areas of the open medullary plate indicate that the tapetum cells are fully localized and separate from the other retinal cells in certain amphibian embryos. The embryos in my experiments had not differentiated the retinal layers sufficiently far to determine with certainty whether there was a persistent disarrangement of the cells, yet the general appearance of the eyes seemed perfectly normal.

2. The median region of the anterior part of the medullary plate cut out, reversed and transplanted in the medullary plate

This experiment is similar to those performed by Spemann on several amphibian embryos. Spemann found that pieces of the medullary plate when cut out and turned around continued to develop with their original orientation undisturbed, thus indicating the early prelocalization of certain future parts of the brain and eyes. When the operation chanced to cut the eye anlage so that part of the future eye material was anterior to the cut and remained in position, while part was contained in the cut-out piece which was then turned around and transplanted, carrying the future eye cells to a more posterior position, two eye regions developed on each side. One arose from the anterior undisturbed cells and the other from the transplanted posterior cells.

The reversed pieces in the present experiment were not long enough to carry the eye back to a distant posterior position, and the cut extended so far foreward that the eye anlage was not divided transversely as in Spemann's operations. The operations were done chiefly to test whether the eye anlage in Amblystoma was well localized and would develop after such reversal of tissues.

Eight embryos were studied after having had antero-median pieces of the medullary plate cut out, reversed and transplanted. Seven of the eight developed both eyes, many of which showed indications of their misplacement. One individual showed one abnormal eye while the other was probably indicated by a doubtful eye structure situated within the archenteron. The general structure of the eyes seemed normal and only slight indication of their reversed origin was shown by a study of these early stages.

Thus if the eye anlage was contained within these cut-out pieces the reversal and transplantation of the pieces did not show any very detrimental effects on the future development of the eyes, though in one of the eight cases one eye was abnormal and the other was greatly misplaced and indistinctly indicated.

The experiments of sticking and disturbing the cells in the anterior end of the medullary plate without actually removing the cells does not prevent the subsequent development of the optic vesicles in an apparently normal manner. Cutting out rectangular pieces of the medullary plate which contain the eye anlage, reversing and replanting them merely cause the eyes to develop in misplaced positions. These two experiments demonstrate the fact that unless the future eye material is well removed by the operation the optic vesicles may later form; this is important in estimating the value of the results in the experiments to follow.

3. *Lateral regions cut from the anterior part of the medullary plate*

Four embryos were operated upon, as shown in figure 1. The indicated region of the right lateral portion of the flat medullary plate was cut entirely away with fine scissors. The area removed did not extend quite to the median line except that in one case it may have or probably did remove tissue beyond the median line. The embryos were killed three days later, cut into sections and studied microscopically.

One of the four lacked both eyes or had eyes so small and poorly formed that they could not be recognized, and the brain showed on one side indications of the operation. Both eyes had thus been removed by an operation which extended at most slightly beyond the median line and certainly did not include the left lateral medullary tissue.

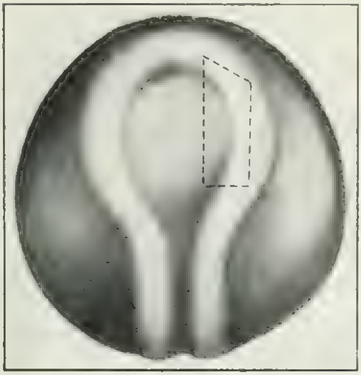
The other three embryos had both eyes present, though one eye in one specimen and both eyes in another were abnormal or defective. In these cases the cut did not extend close enough to the median line to remove all of the eye substance of that side; yet if the eye anlagen at this time had been lateral in position, one would have been completely removed.

A similar experiment was performed on seven somewhat earlier embryos. In these cases where the medullary plate was younger and wider in extent the removed area was confined to a lateral position and did not extend close to the median line, yet it extended laterally into the medullary fold (fig. 2).

Three days later the embryos were killed. On studying the sections of the head region *six of the seven embryos were found to possess two perfect eyes of normal proportions.* The seventh individual had only one well defined eye while the other eye was absent.

The removal of this lateral area of tissue which has been considered the position of the early eye anlage by certain investigators gives no effect on the development of the future eye unless the cut be made to extend very close to the median line. *In eleven operations of this type nine individuals did not suffer the loss of either eye,* while one specimen lacked both eyes and another one had only one eye. The case in which both eyes were absent might have been due to the fact that the cut extended a little way beyond the median line and removed cells destined to form the material of both eyes. Experiments recorded below would indicate that this was the case. A second possibility is that the operation so weakened the individual - which may have been below normal in vigor, though apparently perfect specimens were selected for all the operations -that it lacked the power to differentiate the eyes from the medullary tissue.

Six of the nine specimens which possessed both eyes showed no effects of the removed material in either the size or form of their eyes. The other three embryos had one or both eyes some-



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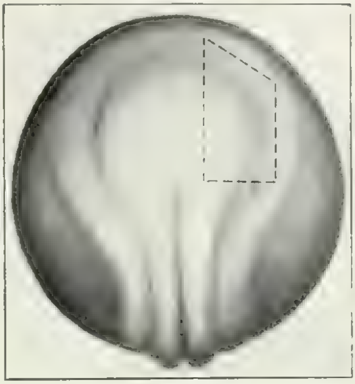

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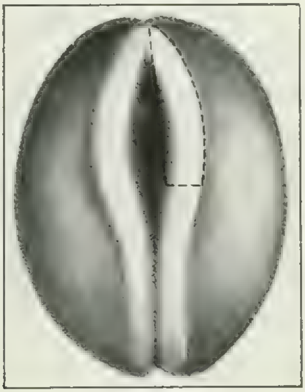

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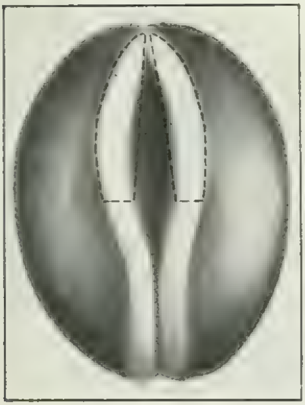

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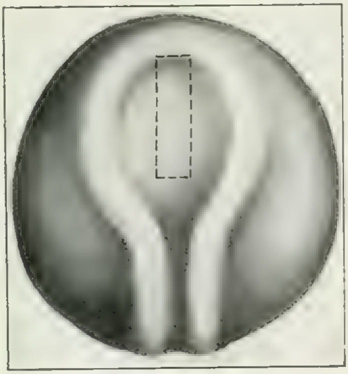

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what smaller than usual. The cases with only one eye defective are perhaps clearly understood on the ground that the cut encroached upon the material destined to take part in the development of this eye. In the cases with both eyes defective it scarcely seems possible that the opposite eye could have been injured by these cuts without entirely removing the eye on the side where the cut was so extensive. The only plausible possibility is that the material for both eyes was medially located and the two eye anlagen closely connected. Any injury to this region might later be shown by both eyes if the injury were at all extensive, and if less extensive and confined entirely to one side of the median line only one eye might show the effect.

I realize that this explanation might be interpreted in part as opposed to Spemann's experiments in which he found the future eye materials so consistently definite in the medullary plate. When the eye anlage was cut transversely and the posterior portion reversed and planted in a distant position it was found that if the forward part of one eye was small the part which had been cut from this and placed posteriorly was large. This fact does not indicate entirely that the eye stuffs are separate; only that when the eye anlage, even if it be median and single, is cut across obliquely and the posterior part removed and transplanted it continues development in a normal consistent manner, the small eye part coming off on the side where less material exists and vice versa.

4. The anterior lateral part of the neural fold and a portion of the medullary wall cut from one side

These experiments were performed on embryos older than those used above in order to test whether the eye anlagen shortly before the coming together of the neural folds might be situated laterally along the border of the folds. In other words, do the eye anlagen primarily arise in the median line or region of the medullary plate and later occupy more lateral positions?

The operation consisted in cutting away one side of the anterior part of the medullary fold and also including in the removed part some of the material constituting the lateral wall of the medullary groove (fig. 3).

Four embryos were operated upon in this manner. Three days after the operation they were killed and prepared for study. A careful examination showed that three of the four specimens possessed both eyes, while one had no eye, or a questionable formation, on the operated side although a perfect eye was present on the unoperated side. Thus in three-fourths of the few cases used, neither of the future eyes had been injured by the operation.

Six other embryos were operated upon in a similar fashion. These were killed two days after the operation. Studying the six embryos in cross section it was found that three possessed both eyes in perfect condition, one individual had two eyes, yet one eye was small and defective on the operated side, and two of the specimens had no eye on the operated side. Thus four of the six specimens had two eyes each, and two of the six had only one eye each.

Combining the results of both experiments it is shown that, *of the ten operated embryos seven possessed both eyes and three lacked an eye on the operated side.* One of the seven which had both eyes present showed a defective eye on the operated side.

A comparison of these experiments with those recorded in the preceding section in which the operation was performed on earlier embryos would indicate that the eye anlage has possibly widened out so as to extend into more lateral regions in these later stages. The weight of evidence, however, would indicate that the position is not directly lateral along the edge or border of the medullary plate, as Spemann has assumed.

It must be remembered that in the folding process which converts the medullary groove into a tube the original borders or edges of the medullary plate come to meet in a middorsal line. After the medullary tube is thus formed the optic vesicles push out from a lateral or really ventro-lateral region and certainly do not in any sense come from the original borders of the medullary plate which are now dorso-median in position.

5. Both anterior lateral parts of the neural fold cut away just before the folds close together

The operation consisted in clipping away with scissors the lateral wall of the neural groove from both sides; that is, the raised folded parts and the dorsal portion of the lateral neural wall indicated in figure 4. The actual crest of the neural folds is of course not future lateral material as in the closure of the furrow to form the neural tube the crest becomes dorsomedian as just mentioned.

Four embryos were successfully operated upon in the given manner. Three days after the operation they were killed and prepared for study. All of the embryos were found to possess both eyes in well developed conditions.

Five other embryos in a similar stage of development were operated upon so as to remove the lateral neural folds as indicated in figure 4.

Four days after the operation the embryos were killed. Studying these embryos in section showed that four of the five possessed both eyes in a well formed condition giving no indication of the injury while the fifth individual possessed only one eye. This last specimen was evidently cut too near the median line on one side so that the future eye material of that side was destroyed.

Thus in nine specimens with both sides operated upon, eighteen operations, only a single eye was missing. The other seventeen operations did not cause any noticeable effect in either the development or nature of the resulting eye.

These experiments clearly demonstrate that the eye anlage is not located along the lateral edge or border of the medullary plate or groove, as Spemann holds. The importance of such facts in connection with opposing explanations of certain eye abnormalities will be fully considered in a following section of this paper.

The next experiments to be presented were performed in order further to substantiate the fact that the eye parts do occupy a median position in the medullary plate or groove. I have been led to think from a study of a number of eye abnormalities that the eye anlage is more or less median in its original position.

6. *The removal of anterior median strips of cells from the wide medullary plate*

In these operations a narrow median strip was cut from the anterior region of the wide medullary plate. The strip in all cases was narrow, being in fact only about one-third or one-fourth the entire width of that portion of the medullary plate lying between the medullary ridges, as shown in figure 5.

Four embryos with wide medullary plates were operated upon in the above manner. Four days later they were preserved for study.

Three of these four specimens were eyeless, showing that the operation had removed the entire eye anlage from the *middle* of the medullary plate. The remaining one of the four embryos had one poorly formed eye while the other was only slightly indicated, so that in this case almost all of the ability to develop eyes had been lost as a result of the operation.

Five other embryos were subjected to a similar operation to that indicated in figure 5 except that the median strip removed was still narrower than in the preceding experiment. These embryos were of different stages but all showed the medullary folds far apart so that the medullary groove was wide open. Three days after the operation the embryos were prepared and studied.

One of the specimens was eyeless, one had two poorly developed eyes, two had one well formed eye while the other eye was questionable or absent, one had both eyes present. Thus the removal of a *very* narrow median strip gave less decided effects than the removal of somewhat wider strips in the experiment above. Yet four out of the five embryos showed eye defects, two having both eyes affected and two having one normal eye and one eye questionable or absent.

Combining the results of the two experiments it is found that the removal of narrow median strips from wide medullary plates exerts the following influence on the future development of the eyes: *Of nine embryos thus operated upon four failed entirely to develop either eye. Two showed two defective eyes. Two individuals developed one perfect and one defective or questionable eye. Only one of the nine embryos showed two apparently normal eyes.*

Since six of the nine embryos had the development of both eyes either entirely suppressed or decidedly affected, and two of the remaining three had one eye affected, it seems most certain that cells destined to take part in eye formation are located in the median region of the medullary plate and are removed by the operation employed. One must conclude that the median optic anlage occupied at least one-fourth or one-third of the width of the medullary plate in the anterior region.

A general statement of the results of certain of the experiments described above may be expressed as follows: Thirty embryos studied after various operations in which lateral portions of the medullary plate were removed at slightly different developmental stages (sections 3, 4, 5) showed in twenty-four individuals, or 80 per cent of the cases, subsequent development of both eyes, while only six specimens or 20 per cent of the cases, showed absence of the eye. In one case the presence of the eye is questionable, in five cases one eye and in one case both eyes were absent. The absence of eyes in the latter cases is possibly due to the cut having been made in a more median position than was intended.

Nine embryos studied after having been operated upon so as to remove a narrow median strip of cells from the anterior portion of the medullary plate (section 6) showed in four cases, or about 45 per cent of the specimens, entire absence of eyes. In four other individuals the eyes were highly defective, one specimen having one poorly formed eye while the other was questionably present. In only one of the nine embryos did both eyes approach the normal condition, from this specimen an extremely narrow piece had been cut away. The optic anlage in this case might have been sufficiently wide at the time of the operation to allow its median portion to be removed and yet enough material remain on either side of the cut to give origin to the two eyes. According to the views of several investigators the removal of this median material should have caused cyclopia, yet it did not. I shall presently attempt to show that cyclopia is not due to the coming together of lateral material in the median line but to a failure of median material to spread laterally.

When the results of these two classes of experiments are contrasted one must conclude that: *The eye anlage in the medullary plate occupies an antero-median position as shown by the various abnormalities incurred when this region is cut away. The failure to injure the development of the eyes in the great majority of cases when the lateral portions of the medullary plate are removed by operation indicates further that the eye anlagen do not occupy lateral positions during this stage of development.*

A DISCUSSION OF PREVIOUS IDEAS REGARDING THE GENESIS OF CERTAIN EYE ABNORMALITIES IN THE LIGHT OF THE ABOVE RESULTS

There has lately been considerable discussion regarding the way in which the cyclopean defect occurs. The experiments described above may serve to concentrate the case on a definite developmental period and, to my mind, settle the question as far as the medullary plate stage is concerned. Whether it is possible to carry any question of vertebrate eye abnormalities, as such, further back in development than this stage seems doubtful, since here it is that the localization of the eye anlage is first known to exist. It is certain, of course, that this anlage does come from cells which are present before the medullary plate has formed. Whether these cells are localized and are entirely future eye anlagen cells, and not indifferent ectoderm cells which might have the power of forming any portion of the brain or central nervous system, one is at present unable to state.

As the case stands, it seems possible to explain the entire genesis of the cyclopean defect from the earliest time at which the optic anlage is definitely known to be localized. This assumption is not made solely from the material presented in the present paper, but from the facts furnished by these experiments together with the observations made upon the large number of cyclopean eyes and brains which the writer has studied during the past several years.

Various authors have at different times thought that cyclopia was due to a fusion of the eyes after they had arisen from the brain. The earlier in development the fusion occurred the more intimately associated the two eye components became. This view has been proven incorrect by actual observation on cyclopean monsters where it is found that the cyclopean condition of the eye, whether large and hour-glass shaped or of small size resembling a normal eye, is present from the earliest appearance of the optic vesicle from the brain. In other words, the several degrees of the cyclopean eye come off from the brain in their final conditions.

The idea of the fusion of the eye parts was deep rooted, however, and now exists in the recent views of Spemann in a refined form. Spemann believes, as several others had previously suggested, that cyclopia is due to an absence of non-ophthalmic tissue in the median region of the medullary plate or groove. This lack of median tissue allows the eye anlagen which he holds to be lateral in position, near the borders of the medullary plate, to come together and fuse in the median plane and later give rise to a cyclopean eye. Cyclopia, according to this idea, occurs in a more or less passive manner, and is, after all, actually a fusion of the eye anlagen of the two sides during development.

I am certain that this fusion explanation which has now been forced entirely back into the medullary plate, is as false as its bolder predecessor which assumed the fusion to take place outside of the brain tissues after the optic vesicles or cups had arisen. Spemann did not advocate this late-fusion view, but claimed from his beautiful experiments on Triton that the cyclopean eye arose out of the medullary tissues in its final condition. He now, however, assumes the rôle of a most ardent supporter of the fusion of the optic anlagen within the medullary plate.

The present writer's opinion of the cause of cyclopia, which was advanced in 1909, was to the effect that this deformity is the result of a weakened development. Spemann has termed this view the 'laming hypothesis,' since my assumption was that the various chemical substances employed in producing cyclopean defects in fish embryos have a tendency to lower the developmental energies and so reduce the power necessary to accomplish the processes involved in the outpushing of the optic vesicles from the brain.

Considering the probable manner in which the cyclopean defect occurs, Adami ('08, p. 241) has theoretically concluded that it is due to developmental arrest or lack of vigor. While I am unable to agree with the details in Adami's argument of a primary growth point at the anterior tip from which is budded off successively the paired parts of the two sides, the anterior ones necessarily arising last after the other parts had been left in more posterior positions, the final conclusion that a weakening of particular developmental processes results in cyclopia is confirmed by all my experiments.

The different types or degrees of the cyclopean defect depend upon the stage in development at which the arrests occur as well as upon the strength or severity of the treatment employed. I shall now attempt to defend this position with the evidence at hand, and in so doing shall as decidedly prove the mistake in considering the defects as the result of any failure to arise of median medullary tissue (other than future eye tissue) and the subsequent fusion of the *lateral* optic anlagen. There is no median tissue between the eye anlagen. The median tissue is the eye anlage itself and will subsequently go to form some portion of the eye, either optic cup or optic stalk, depending largely upon its position and the extent of normal development attained.

The writer had ('07, '09, '10) recorded a number of eye conditions which are considered to be different degrees of cyclopia using the term in a general sense. At any rate, these several conditions differ only in degree and grade perfectly into a continuous series. There is no qualitative difference between them. Spemann has objected to including among these defects certain modified types which are at least closely related to the series in manner of origin, though they may not actually intergrade. (We are now considering only the cyclopean series, not the monster monophthalmica asymmetrica which will be dealt with later.)

One finds on referring to my paper of 1909 (p. 293) that the cyclopean series, A to G, passes from the normal individual through different degrees of association of the two eyes to a median cyclopean eye only as large as one normal lateral eye, then to a cyclopean eye of smaller dimensions until it is extremely minute and may finally be deeply buried beneath the brain as a small pigmented vesicle, as is shown in figure 52, page 321. Only one step further and the eyes fail to arise entirely so that eyeless individuals exist which with a slightly greater power of differentiation or more developmental energy might have given cyclopean monsters. The last assumption is warranted since these eyeless specimens actually resemble the cyclopean monsters in other structures; for instance, the mouth is a narrow proboscis similar to that in the cyclopean monster instead of the usual laterally spread mouth of the normal embryo.

Why should every step and gradation in this series exist if several, or any of the conditions are of a different quality or type? It seems certain that one examining the large number of cyclopean fish on which my study was based would be forced to admit the correctness of the statement that these individuals exhibit different degrees of one and the same defect.

The question then follows, if cyclopia were due, as Lewis, Spemann and others assume, to a failure to develop of median medullary tissue so allowing the eye anlagen to come together in the median plane and fuse, why is not every cyclopean eye equal in mass to the two normal eyes fused? Spemann does not suggest in any place that eye material also fails to arise. He shows in his recent experiments that the future eye is fully laid down in the medullary plate. Not only is the eye present in the medullary plate but the cells destined to form different layers are distinct. Spemann found that certain cells cut out of the medullary plate and planted in more posterior positions formed only the tapetum nigrum layer. If the eye is thus so definitely predetermined in the medullary plate and Spemann believes that cyclopia is due to failure of median medullary cells other than future eye cells then cyclopean eyes ought always to be large or double in size.

The fact is that these predetermined eye cells in the medullary plate in most cases of cyclopia are incapable of perfect differentiation on account of insufficient energy, so they remain in the brain, or only part of them is capable of differentiation and thus small defective cyclopean eyes result.

The actual 'Lähmungs' or suppression is of the eye material itself. *Cyclopia is an eye defect*, and an injury of the eye forming material is the cause. The brain may also be defective as an accompaning abnormality, although in some cases the brain, with the exception of the optic tracts and parts, may be structurally and functionally perfect, as is indicated by the normal life and behavior of many of the cyclopean Fundulus embryos as well as by the existence of the huge cyclopean ray, Myliobates noctula, reported by Paolucci in 1874.

The chemical substances employed by the author in producing the cyclopean defect and a number of others with which McClendon has obtained similar results, all tend to suppress or arrest the development of the eye material in the brain. This future eye material is assumed to occupy a median position. When the arrest is complete, and necessarily taking place at early stages, no eye parts arise from the brain nor are any differentiated within the brain substance itself. Thus a completely eyeless individual is produced.

Spemann states that the eye is capable of differentiation even though it be contained within the brain substance as he has found in amphibia and as Mencl recorded in a teleost. It must be realized that these are *exceptional cases*. A certain amount of energy is necessary for differentiation of the eye to take place even within the brain and when only this amount of energy is present the eye may differentiate within the brain, but when the required energy for any reason is not available the eyes are incapable of any differentiation. Many eyeless individuals have been observed in my experiments which have no indication whatever of eye parts within the brain. Could any one ask, whether this be due to the failure of non-ophthalmic parts of the medullary plate to arise or, on the other hand, to a failure of the future eye forming cells to arise, or to differentiate after they have arisen?

It is not meant to convey the idea that all eyeless brains are related to the cyclopean series, as this is not the case. The future eye forming cells may in some cases have been absent from the start. In other specimens the future optic vesicles might have been in positions to arise normally and laterally and for some reason were incapable of outpushing or of differentiation.

Certain eyeless brains, however, such as those in individuals having the proboscis-shaped mouth, do belong to the series and must be caused in the same way as are the various cyclopean conditions. They have merely responded to a more exaggerated degree.

The most extreme cases of cyclopia with actual eye structures are those in which a small pigmented vesicle arises from a ventromedian part of the brain, as is shown in my figure 52, 1909. This pigment layer of the retina seems to be its most persistent portion, as it may appear when all other recognizable retinal parts fail to arise. There is a possibility that the small tapetum nigrum groups of cells which in some cases formed from Spemann's transplanted portions of eye anlagen may not be due to the fact that only the anlagen of such cells were transplanted, but that all other retinal cells except these were incapable of differentiation when so small a piece of future eye tissue was isolated by the operation.

The next degree of cyclopia is exhibited by an individual having a median eye that is much smaller than one normal lateral eye. We then have a median cyclopean eye of about the same size as one normal lateral eye. The latter case has been termed the 'perfect' cyclopean condition.

All these abnormalities are best explained as follows: The future eye forming cells occupy a median position in the medullary plate and the cells destined to form the two eyes are arranged in one group. This median group of future eye cells normally widens or spreads laterally while two centers of active growth become established which gradually assume more lateral positions until they push out as the two optic vesicles. In the degrees of cyclopia mentioned in the preceding paragraphs the median eye anlage does not widen or spread laterally but is arrested in its primary condition; thus the two growth centers are not sufficiently separated and only a single center exists, and even more than this, the arrest is to such an extent that the entire or normal amount of optic material does not differentiate. Hence, one finds a median cyclopean eye consisting of an amount of eye material far below that normally present.

Other individuals are found in which greater masses of eye material have succeeded in differentiating, and development has been vigorous enough to allow the early eye anlagen to spread to a greater or less degree and establish the two eye forming centers. Such specimens finally present a cyclopean eye showing distinctly its double composition, the two retinae are more or less distinct and the eye large consisting of a greater amount of material than a normal lateral eye, yet less bulky as a rule than the sum of the two normal eyes of the species.

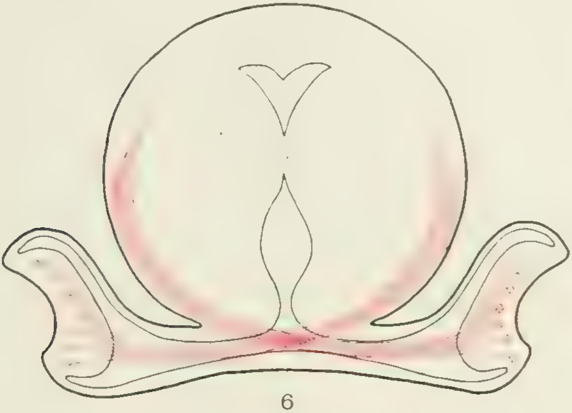
The hour-glass eye or incomplete cyclopia is commonly observed in the experiments. This case is due to a later or less complete arrest in development than those mentioned above. Both eyes have differentiated out of the medullary tissue but the embryo was not vigorous enough to permit their normal separation and outpushing. Thus the eyes come off from their original ventro-median position and remain in close contact or actual union.

Finally one observes individuals in which the eyes are separate and distinct yet unusually close together. Such embryos are able to differentiate their eye material and this material is capable of pushing out from the brain but a slight weakness or arrest has occurred on account of which the optic stalks are short and the optic cups are unable to take a normal position so that they remain unusually near together and look in an abnormal direction.

In the ordinary individual the future eye forming material is first located in a median position in the medullary plate. This material becomes more extensive or widens laterally, and two growth centers are established, the material between the centers finally becomes the median ventral layer of cells of the optic stalks. Later the incipient optic vesicles begin to evaginate or push in a ventro-lateral direction and finally turn dorsally and laterally to reach their usual places at the sides of the head. The optic stalks, however, still lead back to the ventro-median position and there in the fish the optic fibers, following the optic stalks as paths, cross and in higher vertebrates form the optic chiasma always in the ventro-median plane below the brain floor and from here the optic tracts proceed to their centers in the brain.

The median position of the optic chiasma outside and below the brain is an important structural fact in the present consideration. Figure 6 is a diagram of a transverse section through an early brain with the optic cups in their usual position. The optic stalks connect with the brain and the median ventral cell layer is actually part of the stalks. As development proceeds the optic fibers arising from the cells in the retina follow along the optic stalks to reach the brain. The investigations onthe development of the optic nerve have shown that the optic stalks become solid and form the supporting paths or neuroglial scaffolding along which the optic nerve fibers grow. The fibers from one retina meet those from the other in the median plane *below* the brain and in the fish the fibers from the two retinae cross directly while in higher forms partial crossing takes place and the optic chiasma is formed outside the brain. Figure 7 is a sketch representing a cross section of the actual condition of the early optic nerves in a fish embryo. This position of the optic cross is only possible if the median tissue be optic stalk tissue.

Suppose, on the other hand, that the eyes primarily originate from lateral medullary tissues and between the two eyes other brain tissue is present. The optic stalks are then attached to the lateral regions of the brain from which the optic vesicles



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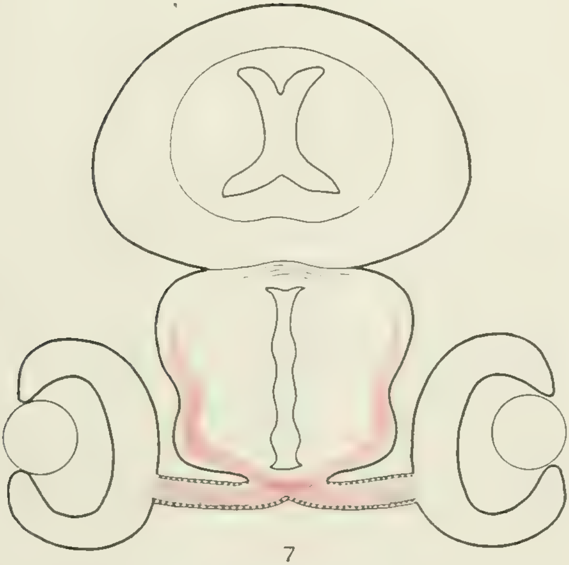

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Fig. 6 A diagrammatic cross section through the brain and optic cups of an early embryo, with all of the median ventral medullary tissue represented as being part of the optic stalks. The future fibers of the optic nerves, shown in red, follow the optic stalks to the median plane where they cross and afterward enter the brain to pursue their course as the optic tracts. The optic cross is entirely beneath and outside the brain.

Fig. 7 A sketch representing the actual outlines of a cross section through the brain and eyes of a late fish embryo. The optic nerves cross below and outside the brain and are surrounded by cells derived from the original optic stalks.

pushed out. Figures 8 illustrates diagrammatically a cross section of this condition. In the course of development the fibers of the optic nerve following the stalk reach the lateral position and must enter the brain and continue within its tissue in order to meet the nerve of the opposite side and form the cross or chiasma. Brain tissue would lie beneath the optic chiasma and the chiasma would necessarily be within the brain. This condition is never found in any normal vertebrate.

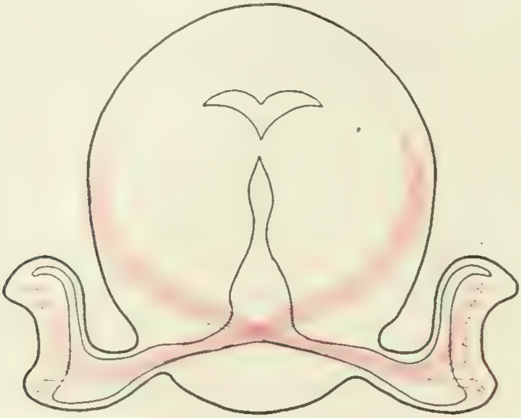

Fig. 8 A diagrammatic cross section through the brain and optic cups in an imaginary case in which the optic connections are lateral with median brain tissue originally lying between the eye anlagen. The future optic fibers growing along the optic stalks as paths reach the lateral points on the brain from which the stalks arose and then enter the brain tissue before having formed the cross. Continuing to grow, the optic nerves meet and cross in the median plane. The cross is within the brain itself and lies above the median mass of tissue which has always existed between the eyes. No vertebrate brain exhibits such a condition.

One might claim that the optic fibers on reaching the brain ran ventrally and formed the cross beneath, but no such change in direction is seen at any stage of their development. The structural relationships seem to depend upon a median origin and connection of the optic stalks.

The fact that a cyclopean eye may have no well formed optic stalk and is entirely median in position not necessitating the absence of any other brain part is in entire accord with the above facts.

It is believed, therefore, that the various degrees of the typical cyclopean condition from eyes unusually close together, to median double or hour-glass eyes, to the large oval eye, to a median round eye of usual size, to the median eye smaller than one normal eye, and finally to complete failure of eye material to arise from the brain are probably all due to developmental arrest. The arrest in development is the result of some influence which has reduced the developmental vigor below the normal so that the energy is not available to carry out the usual processes of differentiation and growth.

The author has figures and described other cases of eye defects which do not fall exactly into the series of cyclopia as considered above; yet these cases are modified conditionswhich are closely related to the cyclopean series both in point of origin and in their final condition. The curtain-like eyes which face the median plane and often have a single lens between them might be considered as delayed cases of cyclopia. The eye anlage widened, the eyes became separated and continued their differentiation, yet they were unable to turn out and assume their normal lateral positions so that they faced in a ventromedian direction with their anterior walls closely approximated as is shown in figure 38 ('09) and figures 1, 4, 5, 6, 13, 14 ('10 a). Such eyes often excite a single stimulus upon a more or less ventral ectodermal region which responds by forming a single lens lying between the two eyes.

The writer has illustrated by a diagram (fig. 15, A and B '10 a,) the difference between these eyes with their choroid surfaces against the lateral ectoderm of the head and their concave retinal surfaces facing medially, and the eyes of a normal individual ('10 a p. 380). As was then stated, the experiments did not give a definite clue to indicate the position of the optic anlagen in the early brain, thus my explanation, or 'laming hypothesis,' referred mainly to the pushing out and lateral development of the optic vesicle and cup. All these cases are decidedly of a type which would suggest lack of developmental energy necessary to attain the normal.

Another group of eye anomalies were extremely common under the same experimental conditions which caused the cyclopean series. These individuals possessed one normal eye in the usual lateral position while the eye of the opposite side in the numerous specimens showed various degrees of imperfection from a condition slightly below the normal in size to complete absence of the eye. Such anomalies were termed 'monophthalmica asymmetrica' in contrast to the symmetrical one-eyed monsters with a median cyclopean eye.

The genesis of the asymmetrical defects is not entirely clear, yet they also are probably due to developmental arrest or suppression of the one eye. The growth centers representing the two future eyes of an individual are rarely equally vigorous and it is frequently noticed that one eye arises slightly before its mate and develops at a little faster rate. It might be that at some critical point in development one of the future eye centers is affected after the growth centers had begun to localize in more or less lateral positions.

In treating the eggs with alcohol a number of embryos occurred in which both eyes were small and defective even though they arose from the brain and attained more or less lateral positions. One might assume this to occur as a result of an arrest in development which affected both eye forming centers after the centers became separate or distinct from one another. Part of the eye forming material is suppressed or its differentiation is prevented so that each eye is decidedly under size and defective. In some of the cases of cyclopia mentioned above the eye was also very small and defective, in these cases the two growth centers which would give rise to the future lateral eyes did not become sufficiently separated so that only a single median eye arose and the reduced vigor permitted this to form only as a small and poorly differentiated structure.

In some instances where an embryo possessed only one member of the normal eye pair this eye was unable to attain its usual lateral position so that it faced the median plane, and if the lips or periphery of the optic cup failed to reach the ectoderm the eye was without a lens.

In none of the specimens, although many were old with the central nervous tissue highly differentiated, was I able to detect any material within the brain which might be considered as representing the missing eye.

Eye conditions such as these are doubtless due to the action of some inhibitory influence which prevents the complete origin and differentiation of eye material, or when it does arise allows it to develop only in a weakened or defective manner. Since monophthalmica asymmetrica occurs so persistently in the same experiments with cyclopean individuals as Lewis, McClendon and I have all found, it is not improbable that the cause is the same in the two cases.

The above considerations have been entirely from the standpoint that the ophthalmic defects under discussion originate during the medullary plate stage. The cause of cyclopia or the tendency to produce such a defect might of course have its origin much earlier in development. It might, in fact, go back to the germ cells themselves or finally it might possibly occur as an hereditary variation. All experimental cyclopia, however, furnishes evidence directly contrary to the latter possibilities. This point I have considered in a previous paper and have clearly demonstrated as have several other investigators that the condition is not due to a germinal variation but may be induced as the result of external stimuli applied during the early development of the eggs.

The cyclopean abnormality may be caused in Fundulus embryos by subjecting the eggs to various chemical stimuli after they have developed normally for as long as fifteen hours. A fifteen-hour Fundulus embryo has the germ ring beginning to form and descend over the yolk sphere, the embryonic shield is scarcely indicated but appears very soon afterwards. Embryos of later stages subjected to the same treatment develop normally, or do not show cyclopia, while stages younger than fifteen hours and as early as the first cleavage are much more readily affected in such a manner as to cause the cyclopean defect. The optic vesicles appear at about thirty hours after fertilization, but the stimulus must be applied at a time sufficiently long before this process occurs, since a number of important steps in eye formation are doubtless taking place before the visible signs of optic vesicles are present.

The fact that cyclopia may be produced after the beginning of the germ ring and embryonic shield in the teleost embryo indicates directly that the defect may occur in what would be the medullary plate stage of amphibian embryos. Thus explanations of the cause of cyclopia must consider it as occurring in the medullary plate stage.

Spemann ('12 b, pp. 38–39) has taken exception to my statement regarding the non-occurrence of cyclopia when eggs are treated later than fifteen hours after fertilization (although the optic vesicles do not arise until about the thirtieth hour) "*since insufficient time exists for the substances to act on the eye anlagen.*" The paragraph following this statement in my paper (p. 388, '10a) is: "The solutions are effective up to a stage in development preceding the formation of the germ ring and embryonic shield, and *the action of the Mg on the eye anlagen probably takes place while the embryonic shield and outline of the embryo are forming.*"[1]

This statement seems to me perfectly direct and clear. Yet Spemann intimates in one sentence that I mean to infer that fifteen hours are necessary for the substances used to penetrate the egg membrane! He then states that I showed in 1907 that KCl would penetrate within a few minutes and stop the embryonic heart beat. The Mg and other solutions used may pass through the membrane equally as rapidly, this I have not fully tested. The fact that it does has no bearing on the *fact* that stimuli do not have sufficient time to *act upon the optic anlagen to induce cyclopia* when applied to the eggs at later periods in normal development than fifteen hours after fertilization since they must act on the anlagen in the embryonic shield, and to act later is too late. The reason for the nonoccurrence of cyclopia when eggs were treated at periods later than fifteen hours after fertilization or fifteen hours before the appearance of the optic vesicles is that the optic anlagen and all other embryonic parts are constantly changing during development and have passed beyond the critical stage. It is not only a question of how quickly one may stimulate but in what condition the anlagen are at the time of stimulation. It is safe to say that cyclopia can *not* be produced after the optic anlagen have proceeded to some definite stage in their normal development. Even if Mg should penetrate all the membranes within a few seconds its action could never induce cyclopia in an embryo with the two optic vesicles visibly formed. There was no question of the time necessary for penetration, but an important question of the embryonic condition to be *acted* upon, the critical condition of the optic anlagen.

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[1] The last clause was not originally in italics.

In the above connection it may be mentioned that such substances as alcohol and ether, when administered to an early embryo may cause it to develop into a decided monster. A late embryo or foetus may respond to the same treatment by producing an individual exhibiting no gross morphological defects yet showing decidedly abnormal nervous reactions, while a similar treatment might exert little or no effect upon a fully formed individual. A stimulus could not cause the mature individual to change into a structural monster. The developmental period of administration is of as high importance in determining the result as is the nature of the stimulus used, unless of course the stimulus be entirely destructive.

Again Spemann misinterprets a statement regarding the action of Mg. It was remarked that the cyclopean embryos developing in the Mg solutions were, except for the eye defects, more perfect than those arising from treatments with alcohol, ether, and so forth. The Mg cyclops often had apparently normal brains, could swim in normal fashion, took food and reacted to stimuli much as normal embryos did, while those embryos treated with alcohol and ether had various defects of the brain and cord. In this connection it was cited as of interest that Mayer had recently found in studying nerve muscle preparations that Mg salts seemed to prevent activity by affecting the muscle directly without apparently affecting the nerve. There is, of course, no direct connection between these facts and cyclopia; the mention was made merely in a general way, and most decidedly did not intend to convey the notion that muscle contractibility and the outpushing of the optic vesicles were phenomena of similar nature. They are similar only in that both are dynamic processes and require energy for their accomplishment.

There is no necessity for further discussing the *fact* that a number of eggs when subjected to the same solution do not all respond in a like manner (Spemann '12 b, p. 37). This is a typical case of differences in individual resistance and vigor which is observed among any one hundred individuals of any living species. It is equally true that the two sides of a so-called bilateral individual are rarely, if ever, identical.

Spemann is no doubt correct in stating that the relationship between cause and effect in my chemical experiments on cyclopia is not clear. Yet it seems to me that it is not entirely dark, the entire relationship between cause and effect in biological experiments is rarely if ever clear step for step.

I should like, however, to point out that the chemical experiments did one thing in proving that cyclopia could be caused from normal embryos through the action of the environment. This fact did away with all theories of germinal origin of the defect, one of which was strongly presented by Wilder about the same time. The experiments also make clear the stage in development at which cylopia may occur, and they further supply the richest amount of material yet available for the study of this defect. Finally, they prove to my mind that cyclopia is a developmental arrest and may be due to any cause which lowers developmental vigor at certain critical stages in the formation of the eye anlagen. These important points in the study of this defect have certainly *not* been made clear by the mechanical experiments though I do not deny that they might possibly have been.

To quote again from Spemann:

> Gegen diese Defekthypothese erhebt Stockard (1909b, p. 172) die Fragen: warum sollte bei den Magnesiumembryonen gerade das Gewebe zwischen den Augen ausfallen, und keine anderen Gewebe?; warum sind die Riechgruben bei Cyclopie manchmal verschmolzen und manchmal getrennt?; . . . . ist bei den asymmetrisch-einäugigen Missbildungen der einseitige Augendefekt etwa auf die Abwesenheit der einen ersten Augenanlage zurückzuführen? —Darauf möchte ich mit der Gegenfrage antworten: sind denn all diese Tatsachen verständlicher bei Zugrundelegung der Stockard 'schen Lähmungshypothese?

I should answer now, as it was inferred above, that they most decidedly are, and an attempt to show this fact in some detail has been made in the preceding pages.

The title of the recent paper by Spemann "Zur Entwicklung des Wirbeltierauges" might better have been "The development of the vertebrate lens," as little or almost no attention is given to other parts of the eye. The problem of the lens formation is very fully considered.

"Cyclopia of the lens" is discussed from the same standpoint as cyclopia of the optic cups. In the case of the lens median ectodermal cells may be missing so that the two normally lateral lenses fuse in the median line. It seems to me that this is the one straw too much for the 'Defekthypothese.' The imagination which pictures the falling out of just the exact amount of median ectodermal tissue which would allow the primary lens forming cells of the ectoderm to fuse towards the center and keep proper pace with the movements and final position of the various cyclopean eyes which my Fundulus material presents must be most vividly active. An even more plausible possibility out of this imaginary dilemma is to consider the lens anlage as a single median group of cells that divides into two parts which come later to lie in lateral positions. This view would at least have the advantage that in cyclopia of the eye "cyclopia of the lens" would maintain the lens forming cells in a fairly median region, and in normal development the lens cells would have a more or less definite path to follow and place to reach. The pineal eye in many forms possesses a fairly definite lens which must have arisen medially and the present lens may have been somewhat more anterior yet also median. These suggestions are of the most speculative nature and are intended merely as such. In my experiments many of the embryos which possess supernumerary lenses show, as Spemann has called attention to, that the accessory lens may actually lie more anterior than the eye. Nevertheless, others, show free lenses in lateral positions.

The presence of a lens in the cyclopean eye is explained by the fact, well established for several species, that an optic vesicle or cup possesses the power to stimulate lens formation from any region of the head ectoderm with which it comes in contact. There would be no necessity of imagining a condition of "cyclopia of the lens" even though a median lens should be observed in an anophthalmous monster. Normal lateral lenses have been observed in anophthalmic monsters (see figs. 1 and 5, and fig. 3, plate II, 1910 b).

The embryo from which my figure 4 ('10 b), was taken is of new interest in connection with a hypothetical case called for by Spemann (p. 81 '12 a). He states as a possibility that

> der cyclopische Defekt nur die Epidermis betrifft, so dass Riechgruben und primäre Linsenbildungszellen median zusammenrücken, während die Augenbecher, wie normal seitlich gelegen, sich ihre Linsen aus der dortigen Epidermis bilden. Durch solche Fälle würden in der Tat an einem und demselben Kopf beide Fähigkeiten demonstriert, die der Linse zur Selbstdifferenzierung und die des Auges zur Linsenerzeugung. Bis jetzt liegen aber derartige Fälle nicht vor.

The case however, was, recorded at the time and seems to fill the requirements set forth. The independent origin and differentiation of the lens is demonstrated in a median position slightly more antérior than the eyes, and the more or less lateral eyes have derived lenses from the ectoderm with which they came in contact as is shown by figs. 9 and 10, 1910 b, yet this ectoderm is part of the usual region from which an optic cup has the power to derive a lens. The power to form lenses, as Speman and I have claimed, is possessed by the ectoderm of the head.

According to Spemann's assumption, the embryo (fig. 4) presents true "cyclopia of the lens." The condition, however, may better be interpreted as an illustration of the high lens-forming capacity possessed by the ectoderm at the anterior tip of the head.

Lens-forming power seems to diminish from the anterior tip of the head backward until trunk ectoderm no longer possesses the capacity to form a lens, as Spemann found in transplantation experiments. For this reason independent lenses arise, as a rule, far anterior to and often in front of the more or less lateral eyes (my figs. 3, 4, 7, 9, 10, 11, 12, 21, etc. '10 b). In fewer cases independent lenses are found in more posterior lateral regions approximately in the normal lateral eye position (figs. 1 and 2 and 3, plates I and II '10 b). Figure 9 may serve to illustrate diagrammatically the extent and gradation of the lens-forming power possessed by the head ectoderm. This rather diffuse localization of lens-forming cells in the general head ectoderm as demonstrated by numerous experiments seems sufficient to account for all phenomena of lens formation in cyclopia, as well as the supernumerary lenses which the writer has reported.

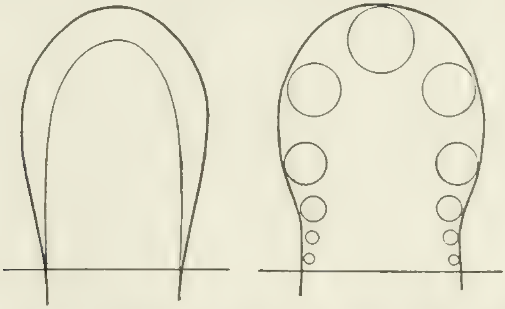

Fig. 9 Two diagrams indicating the primary lens-forming power of various portions of the head ectoderm. The lens-forming tendency is considered to be greatest at the anterior end and gradually decreases towards the trunk ectoderm until the ability to form a lens is lost where the trunk begins. The rows of circles indicate the magnitudes of lens-forming tendency in different regions and do not signify the size of the lenses. Posterior lenses may be as large as anterior ones, yet they occur less frequently as independent structures. Free lenses usually occur near the anterior tip.

The median position in cyclopia of normally bilateral organs involves one other part. The nose or nasal pits in the cyclopean fish are sometimes median and single, or occasionally bilateral and more or less normal in position. I might also add that the nasal pits are often absent. Again the 'defect hypothesis' must expect median cells to fail and so allow the two nasal pits to fuse medially. In this case the evidence is still stronger for the primary median origin of the ectodermal anlage. Dohrn's studies on Ammocoetes have shown the median position and relationship of the nasal and hypophyseal invaginations. The question of monorhiny in the cyclostomes is not fully determined but evidence is certainly available to indicate a median nose anlage. These, however, are phylogenetic considerations which would only serve to prolong the present discussion.

The experimental results presented by Spemann relative to questions of lens formation agree almost entirely with the conclusions which were presented in my paper of 1910 b. He disagrees, however, with many of my interpretations, yet I believe the disagreement is not as complete as it often seems.

I had suggested that the power of the ectoderm to form a lens without the presence of an optic cup was less vigorous or efficient than when the optic cup combined its stimulus with the tendency to lens formation possessed by the ectoderm. For this reason when the ectoderm was injured by many of the mechanical operations which have been employed in the study of lens formation the injured ectoderm was unable to form independent lenses although normally it would have had such power. Attention was called to the different results of Lewis and Miss King on Rana palustris.

Spemann ('12 a, p. 49) rejects this idea although he produces evidence in his paper to prove its very probable correctness. Compare the results he obtained in the origin of independent lenses after cutting out the optic anlagen from medullary plates with glass needles, in which case the ectoderm in the primary lens-forming region was uninjured, with the results following a burning out of the eye anlagen with hot needles, in which case neighboring tissues were necessarily injured. After the latter operation only *one well formed lens occurred in five cases.* A further comparison between the glass needle operations on early stages with open medullary plates and later stages where the ectoderm was raised and the optic vesicles cut out from beneath it show a difference in the response of the ectoderm in producing free lenses in favor of the less injured or less disturbed ectoderm.

A few of the early lenses figured by Spemann are at least questionable. For example, figure 44 ('12 a) shows on the operated side an irregular thickening within a single cell layer. Such a thickening might readily have resulted from the disturbance to which the portion of ectoderm had been subjected. This specimen is cited as evidence that the accessory or supernumerary lenses in my experiments may have arisen as buds from a common origin. The possibility of early lenses constricting or budding into two or more is freely admitted. Figures 14, 17, 18 and 20 ('10 b) show constricted or double lenses. If the constriction had been carried further two lenses might have resulted. Yet the relative positions occupied by several other of the lenses figured are difficult to account for on the above basis.

Finally, there is no question of the fact that in numbers of the fish monsters which I have figured and described small and ill-formed eye vesicles or fragments are associated with large well-formed lenses. The fact of the constant association of such optic structures with lenses whenever the optic parts chance to lie near the ectoderm makes it practically certain that the defective eyes have stimulated the lenses to arise in these positions. The size of the lens in Fundulus is not regulated by the size of the optic cup. This is further proven by the small lenses in large eyes and by large protruding lenses in rather well-formed but small eyes. The error in logic which Spemann ('12 a, p. 81) claims to exist on page 405 of my 1910 b paper in discussing these eye fragments I am unable to detect and several other embryologists have been unsuccessful in pointing it out.

SUMMARY

1. Experiments in which certain regions are removed by mechanical operations from the medullary plate of Amblystoma punctatum seem to show that the earliest optic anlage is median in position.

Thirty embryos from which lateral portions of the medullary plate and the anterior lateral part of the medullary fold were removed at slightly different stages gave in twenty-four cases, or in 80 per cent of the individuals, subsequent development of both eyes. In five individuals one eye was absent and in one specimen both eyes failed to arise. The absence of eyes in the latter cases was probably due to the cut having been made in a more median position than was intended.

Nine individuals were operated upon so as to remove narrow strips of cells from the anterior median portion of the medullary plate. Four of these cases, or about 45 per cent of the specimens, failed entirely to develop eyes. According to Spemann and others they should have given some degree of cyclopia. Four other individuals possessed highly defective eyes, one embryo having one eye poorly formed while the other was questionably present. Only one of the nine specimens so operated upon was capable of developing both eyes to an extent approaching the normal.

2. When the cells in the anterior portion of the open medullary plate are disturbed by being stuck and scraped in various ways with steel needles they do not loose their power of giving rise to optic vesicles and cups which are normal in appearance during the early stages, later stages were not studied.

3. If the optic anlage be cut out of the medullary plate and reversed in position and then transplanted in the medullary plate it still retains the power to give rise to optic vesicles and cups which are abnormal in position to an extent depending upon the distance the anlagen were shifted by the operation.

The facts furnished by these experiments are considered in connection with recent views regarding the genesis of certain ophthalmic defects.

LITERATURE CITED

Adami, J. G. 1908 Principles of pathology, vol. 1, p. 241, Lea and Febiger, New York.

Dohrn, A. 1883 Studien zur urgeschichte des Wirbelthierkörpers III. Die Entstehung und Bedeutung der Hypophysis dei Petromyzon planeri. Mitt. Zool. Stat. Neapel., Bd. 4, pp. 172–189.

King, H. D. 1905 Experimental studies on the eye of the frog. Archiv f. Entw.-Mech., Bd. 19.

Lewis, W. H. 1909 The experimental production of cyclopia in the fish embryo (Fundulus heteroclitus). Anat. Rec., vol. 3, pp. 175–181.

Mayer, A. G. 1909 Rhythmical pulsation in Scyphomedusae. Carnegie Institution Pub., no. 102, pp. 113–131.

McClendon, J. F. 1912 An attempt toward the physical chemistry of the production of one-eyed monstrosities. Am. Jour. Physiol., vol. 29, pp. 289–297.

Mencl, E. 1903 Ein Fall von beiderseitiger Augenlinsenausbildung warhrend der Abwesenheit von Augenblasen. Arch. f. Antw.-Mech., Bd. 16.

1908 Neue Tatsachen zur Selbstdifferenzierung der Augenlinse. Arch. f. Entw.-Mech., Bd. 25.

Paolucci, L. 1874 Sopra una forma mostruosa della myliobatis noctula. Atti della società Italiana di Sc. Naturali, tom. 17, pp. 60–63.

Spemann, H. 1912 a Zur Entwicklung des Wirbeltierauges. Zool. Jahrb., Bd. 32. Abt. f. allg. Zool. u. Physiol., pp. 1–98.

1912 b Über die Entwicklung umgedrehter Hirnteile bei Amphibienembryonen. Zool. Jahrb., Suppl. 15. (Festschrift für J. W. Spengel, Bd. 3) pp. 1–48.

Stockard, C. R. 1907 The artificial production of a single median cyclopean eye in the fish embryo by means of sea-water solutions of magnesium chloride. Arch. f. Entw.-Mech., Bd. 23.

1909 The development of artificially produced cyclopean fish, "The magnesium embryo." Jour. Exp. Zoöl., vol. 6, pp. 285–338.

1910 a The influence of alcohol and other anaesthetics on embryonic development. Am. Jour. Anat., vol. 10, pp. 369–392.

1910 b The independent origin and development of the crystalline lens. Am. Jour. Anat., vol. 10, pp. 393–423.

Wilder, H. H. 1908 The morphology of cosmobia. Am. Jour. Anat., vol. 8, pp. 355–440.

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THE ARTIFICIAL PRODUCTION OF EYE ABNORMALITIES IN THE CHICK EMBRYO

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TWO PLATES

During the springs of 1911 and 1912 a series of experiments were conducted on hens' eggs aiming towards a definite modification of development so as to produce typical defects. A large number of eggs was used and numerous methods of treatment with various chemical stimuli were employed. The results, however, have not been of a definite nature, nevertheless they do indicate a decided tendency on the part of the developing central nervous system to respond to certain classes of stimuli in rather typical fashions. The responses are not in any sense specific for a given treatment but the same rather definite response may be obtained by a number of methods. This statement applies equally to other experiments on the artificial production of definite defects in the embryo. The earlier view that these defects were specific responses to the given chemical substance employed as has been advocated by Herbst,[1] Hertwig, O.[2] the writer[3] and others is no doubt erroneous.

The important fact, however, is that a certain definite response on the part of the developing organism may be consistently obtained after carefully adjusted treatments with a large number of different substances. Since the response is the same in each case it is very probable that the substances though widely different act similarly on the embryonic organism, for example, in certain cases they may serve simply to lower the developmental metabolism and thus prevent or arrest the formation of particular structures.

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[1] Herbst, C. Experimentelle Untersuchungen, u. s. w., Zeitschr. f. wissensch. Zool., 4, 1892; Mitt. a. d. Zool. Staz, zu Neapel, 1893; Arch. f. Entw-Mech. 4, 1896.

[2] Hertwig, O. Urmund und Spina bifida. Eine vergleichende morphologische teratologische Studie an missgebildeten Froscheiern. Archiv f. Mikr. Anat. Bd. 39, 1892; Die Radiumkrankheit tierischer Keimzellen. Ein Beitrag zur experimentellen Zeugungs- und Vererbungslehre. Archiv f. Mikr. Anat. Bd. 77, Abt. 2, 1911.

[3] Stockard, C. R. The artificial production of cyclopian monsters: The "Magnesium embryo." Jour. Expr. Zool., 6, 1909.

The hen's egg readily lends itself to chemical and mechanical experiments and has been largely employed in experimental teratology. It has long been known that by running the incubator at too high or too low a temperature or by reducing aeriation by varnishing the shell one is able to obtain a most varied group of monsters. Féré[4] has used a great number of methods to produce monster chick embryos. In 1899 he treated eggs with alcohol fumes before incubation and found that the fumes penetrated the shell and produced various abnormalities in the embryos. Féré[5] also repeated Preyer's[6] experiment of removing the egg from the shell and allowing it to develop in glass dishes. Preyer was only able to keep the eggs under observation in this manner for two or three days, while Féré devised a better means of ventilation and succeeded in keeping the eggs alive for six days. Many of the embryos developing out of the shell showed abnormalities. Féré's reports merely record the experiments and mention the types of monsters obtained but no detailed or systematic study was undertaken and his experiments have generally passed unnoticed. One must, however, appreciate the rather ingenious and various methods of treatment which Féré employed.

The experiments to be briefly described in the present communication are presented in order to show that the central nervous system and the eyes of the chick embryo become affected in a manner closely similar to that which I have recorded for the fish embryos when treated with alcohol, ether and other substances.[7]

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[4] Féré, Ch. Influence du repos, sur les effets de l'exposition préalable aux vapeurs d'alcool avant l'incubation de l'eouf de poule. Compt. rend. Soc. de biol. 51, 1899.

[5] Féré, Ch. Remarques sur l'incubation des oeufs de poule privés de leur coquille. Compt. rend. Soc. de biol. 52, 1900.

[6] Preyer, W. Physiologie spéciale de l'embryon. Trad. franc, p. 16, 1887.

[7] Stockard, C. R. The influence of alcohol and other anesthetics on embryonic development. Am. Jour. Anat., 10, 1910.

Hens' eggs were exposed for different lengths of time to the fumes of alcohol and ether. The eggs used in the experiments had been laid for only two or three days. Shallow dishes were arranged with a wire screen bottom beneath which absorbant cotton soaked with 95 per cent alcohol was placed. Eggs were placed upon the wire screen and the dishes covered and left standing at the room temperature. During the two years several hundred eggs were treated in this manner.

After the eggs have been exposed to the fumes for a short while the shell becomes covered with moisture, the condensed alcohol vapour and this vapour penetrates the shell. The eggs were exposed for from twenty minutes up to thirty hours at room temperature. The shortest exposure that gave effects was three hours and forty-five minutes, though in many cases an exposure of as long as eight hours was non-effective. Exposures of from fourteen to twenty hours gave the best results. In these cases almost every embryo was abnormal yet most of them were able to continue development for several days at least. Exposures of twenty-three hours or more were usually fatal, the eggs failing to develop after being put into the incubator.

The chief point to consider in the amount of exposure is the temperature. When the temperature is high evaporation is more rapid and more alcohol enters the egg in a given time.

If eggs are placed in the incubator immediately after the treatment liquid oozes out of the pores in the shell on account of the slight expansion of the egg contents as the temperature rises. A certain amount of the alcohol is no doubt lost by this process. It is better, therefore, to allow the eggs to remain at room temperature for several hours after being removed from the fume dishes and before being placed in the incubator. Féré found that eggs put into the incubator immediately after treatment with alcohol fumes were not so decidedly affected as those treated for the same length of time but not subjected to the raised temperature until several hours after the treatment.

In other cases eggs were exposed to the alcohol fumes while in the incubator. Weak alcohol solutions were placed below the egg tray and evaporated slowly. This treatment was also continued for different lengths of time and in many cases gave more decided effects than those obtained by the treatments before incubation.

Ether fumes were also employed in the above manner. These fumes induce the same general types of developmental abnormalities though they are more decided in action than alcohol fumes and kill the embryos much more readily.

Several injection methods were used and a number of substances were injected into the egg but the results were indefinite and often negative. In many cases the injection was a failure in that it either coagulated the albumen in the region, or injured the egg so that it did not develop.

Following effective treatments with the fumes of alcohol or ether the embryos were found to be small and behind the control in their rate of development. The abnormalities most abundant were of a general nature, in some cases the entire body of the embryo was absent while the area vasculosa was present containing blood islands and embryonic vessels. Other cases showed small embryos with the brain portion of the neural tube poorly developed. The circulation in many of the embryos was slow and sluggish and in such cases hydramnious conditions were present and the blood sinuses were also distended.

A number of the embryos showed various abnormal eye conditions and these are the defects of particular interest since exactly similar abnormalities have been gotten in abundance when developing fish eggs are subjected to the actions of ether and alcohol. In several experiments embryos occurred with small poorly formed eyes which closely resembled the minute defective eyes most commonly found in alcoholized fish embryos.

A few typical cyclopean conditions were obtained showing different degrees of the defect. However, never more than three or four per cent of the embryos showed cyclopia even in the most successful experiments and in most instances cyclopia did not occur at all. Nevertheless, it is of importance to find that these treatments do occasionally induce the same variety of defects in the chick as was so abundant in many of the fish experiments.

The monster monophthalmicum asymmetricum, that is, an individual with one eye of the normal pair perfectly developed and the other eye either absent or defective to a marked degree, was commonly seen in the different groups of embryos, plates 1 and 2. This condition was more often found than cyclopia, yet it also was not as abundant as in fish embryos developing in solutions of alcohol or ether.

The failure to obtain definite defects in large numbers in the chick embryos is no doubt due to the fact that the amount of treatment is much more difficult to regulate than in such an egg as that of the fish. The great variation in the size of hens' eggs, the amount of albumen as well as yolk, the thickness of the shell, etc., makes it almost impossible to treat a number of eggs to the same degree. The treatment must of course be delicately balanced in order to obtain such typical defects as cyclopia and monophthalmica since they only occur as responses to a certain injury or arrest at a critical developmental stage.

It has also been found in a series of experiments which is being conducted to test the effects of alcohol and ether on the structure of the offspring from guinea-pigs that a completely eyeless young animal was produced and the nervous systems of almost all the offspring show some defects due to the treatment.[8]

During the winter of 1912 one of the incubators in the laboratory was placed in a room into which a ventilation system opened. The same system communicated with rooms in the chemical laboratory and fumes conveyed by the ventilator although rarely noticeable in odor were sufficient to injure the developing chicks. Many of the embryos died during early stages. The eggs were being used in tissue culture experiments by Dr. Burrows and were usually opened after having developed for about twelve to eighteen days. Several of these large chicks were found to have only one lateral eye. They were similar to the early embryos formed in the above experiments and were asymmetrical monophthalmic monsters identical with those I have described in fish embryos. Photographs of three of these large chick embryos are figured in plates 1 and 2, since they illustrate the defect far better than the young three and four day twisted embryos. The fumes injured the eggs and caused the same types of developmental arrests or supression as are obtained with the other substances discussed above. After the incubator was removed from this room the eggs in it developed in a perfectly normal manner.

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[8] Stockard, C. R. The effect on the offspring of intoxicating the male parent and the transmission of the defects to subsequent generations. Am. Naturalist, vol. 47, 1913.

These structural deformities and their experimental production are recorded to emphasize the general nature of such defects and their wide occurrence among different types of embryos when treated with any substance which tends to arrest development or lower their developmental rate and vigor. Elsewhere[9] I have attempted to show how all abnormalities such as these eye structures may be explained merely as developmental arrests. Thus their wide occurrence in spite of their typical appearance.

PLATE 1

EXPLANATION OF FIGURES

Three views of an asymmetrical one-eyed chick monster which occurred in Dr. Burrow's incubator. The upper photograph shows the eyeless side, a small nodule of skin in the orbital depression represents an abortive eye-lid formation. The lower left figure represents the opposite side with a perfect eye, the fully developed lids are closed. The lower right figure giving a dorsal view of the head emphasizes the general asymmetry due to the absence of the one eye. The beak is permanently crossed since the upper jaw is forced to incline towards the eyeless side while the lower jaw remains in a normal position. It is thus impossible to close the beak as all the figures show.

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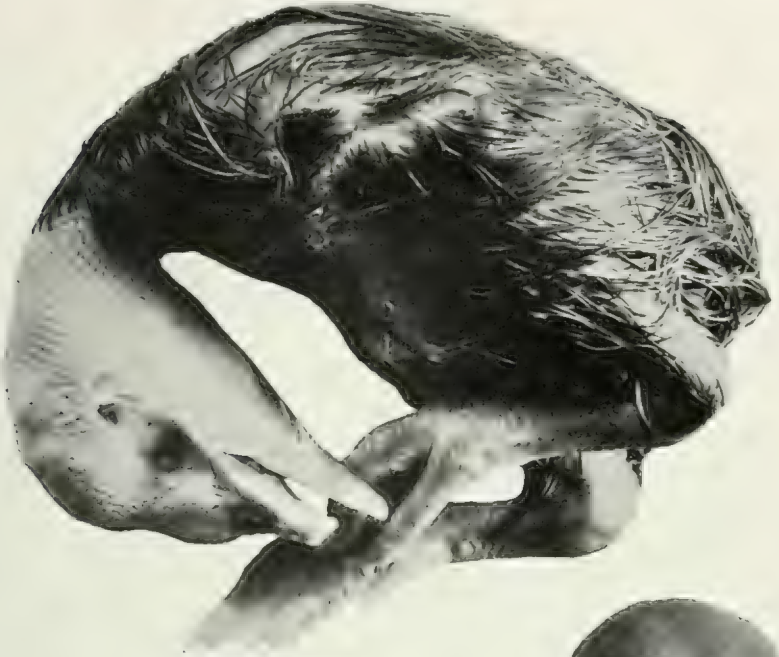
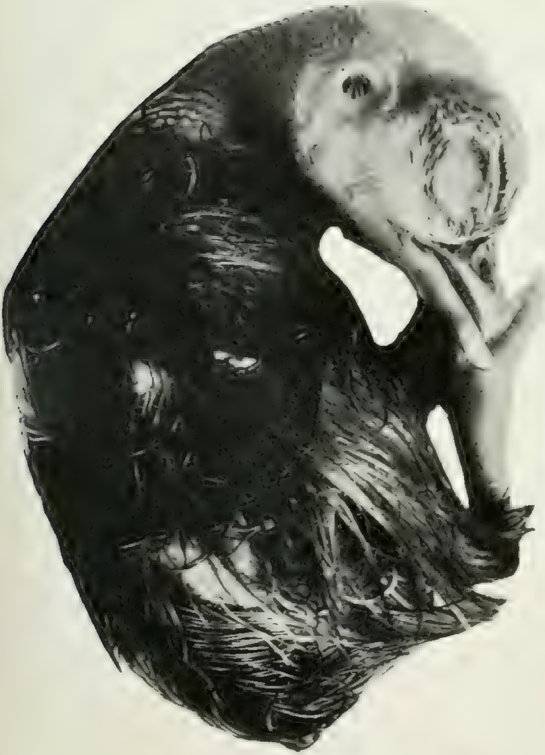
[9] Am. Journ. Anat., vol. 15, no. 3, 1913.





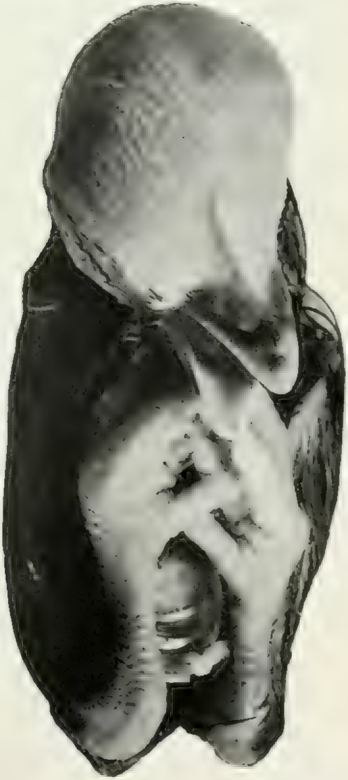
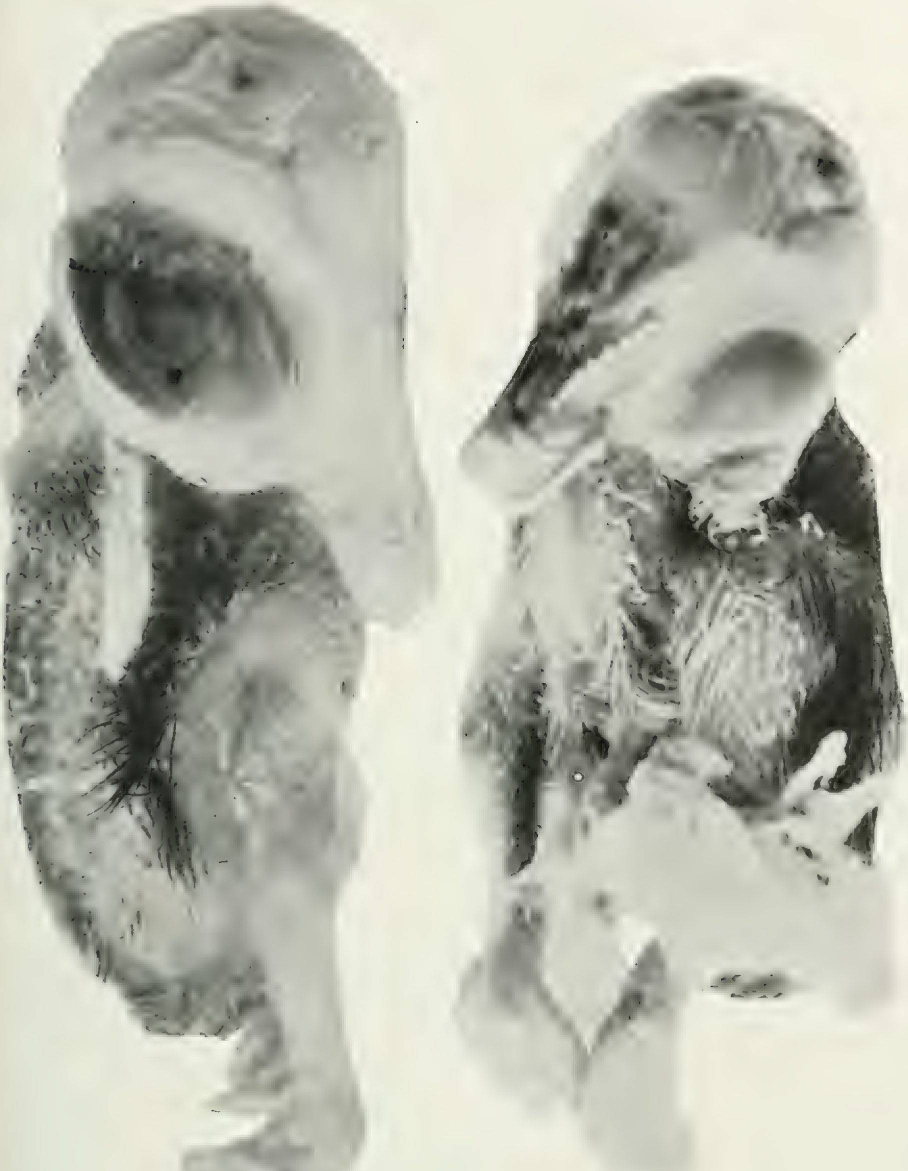


PLATE 2

EXPLANATION OF FIGURES

Two other specimens of monster monophthalmicum asymmetricum. The huge eye of the embryo chick is seen on one side of the head while the other side is eyeless. Both of these embryos also show the twisted upper jaw and the permanently open condition of the mouth.



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THE ARTIFICIAL PRODUCTION OF STRUCTURAL ARRESTS AND RACIAL DEGENERATION

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Various chemical and physical means are often experimentally employed to modify development. In fact the development of an animal embryo in almost any unusual or abnormal environment causes the embryo to respond by deviating from the normal path of development to a degree depending upon the extent of the environmental modification. The same method of treatment does not cause every embryo to respond in a like manner, so that when a number of developing eggs of a given species are subjected to certain chemical stimuli numerous types of defects result. It has been possible, however, to regulate the treatment in certain cases so as to induce a rather definite or consistent response on the part of the embryo.

The developing eggs of two species of fish when subjected to the actions of various magnesium salts, alcohols, ether, etc., have been found to respond by giving rise to large numbers of cyclopean monsters. This response is oftentimes most consistent, so that sixty in one hundred individuals will be of the cyclopean type. Other defects of the eyes and central nervous system are also caused to occur in these solutions. All of the eye conditions may be interpreted as arising through developmental arrests. The abnormal environment tends to lower the developmental vigor of the embryo and this lack of vigor is particularly shown in the condition of the eyes, since the budding off and outpushing of the optic vesicles are apparently the most extensive growth phenomena taking place in the early embryo.

The embryos treated with alcohols, etc., often had abnormal brains and in addition to cyclopia, which was the most frequent eye defect, many were completely eyeless, while others had one eye of the pair fairly well developed while the opposite eye was small and defective or at times entirely absent. Such conditions were termed monophthalmica asymmetrica.

The eggs of the common fowl may be treated with the same substances as those above mentioned. The developing embryos respond in a somewhat similar manner, though the response is not so consistent as in the fish. When hens' eggs are placed in an atmosphere saturated with the fumes of alcohol, within a short time, from one to three hours, enough fumes penetrate the shell and act on the early embryo to cause it to develop abnormally. Many of the affected chicks showed eye conditions similar to those produced in the fish. Cyclopia was not uncommon, and the monophthalmic asymmetrical monsters were often produced.

The cause of these defects in the chick is thought to be the same as in the fish. The fumes acted to weaken or injure the embryo so that it no longer possessed sufficient developmental energy to give rise to normal fully formed optic vesicles. In some cases the arrest prevented the lateral migration of the vesicles so that they arose in a ventro-median position giving rise to cyclopean monsters. Other individuals were able to separate the eye anlagen, yet one of the anlagen failed to differentiate normally and so formed an imperfect eye or was entirely suppressed, thus no eye or a defective eye occurred on one side. Still other specimens were completely unable to differentiate or develop the eye parts and gave rise to eyeless chicks.

It is thus seen that in fishes and birds the experimenter is able to regulate the type of abnormality to some extent at least. The eggs of these animals develop externally; that is, outside of the maternal body. Such eggs may be directly treated with various substances. The eggs of higher mammals develop within the mother's body and the proposition of treating them with chemical or physical stimuli is thus complicated. The question arises whether the effect is due to the action of the substance used upon the embryo directly or whether the effect is secondary and due to the changed condition induced upon the mother by the treatment.

It is found that volatile substances such as alcohol and ether may be employed with the least complication in their manner of action. When alcohol is given by inhalation to guinea pigs it is readily taken into the animals' blood and produces a state of intoxication. Guinea pigs given alcohol in this way six times per week have maintained their bodily vigor, fattened and lived in an apparently healthy state for as long as two and one half years.

Other investigators have found that alcohol given to pregnant guinea pigs passes directly into the blood and tissues of the fœtus. It has also been shown that alcohol has an affinity for the reproductive glands and may be demonstrated in the seminal fluid of the male shortly after being administered. Thus the spermatozoa may practically be bathed in a solution of alcohol, and the early embryo of a mammal may be subjected to the action of alcohol almost as directly as the embryo of the fish or hen considered above.

The guinea pigs were first tested by normal matings and found to produce vigorous offspring. Matings of the alcoholized guinea pigs were then made in various combinations. Alcoholic males were paired with normal females. This is the paternal test and is the only true test of the action of the substance on the germ cells. In such matings the egg is from a normal female and develops in a normal body, hence any defect in the offspring is probably due to the action of the alcohol on the spermatozoon.

Treated females were paired with normal males, the maternal test; in this mating there are two chances of affecting the offspring. The alcohol may have acted on the unfertilized ovum so as to modify its nature and cause it to develop into an abnormal individual or secondly the ovum may be normal but its development within a mother with alcoholized blood may result in a defective guinea pig. In any case we would be unable to differentiate between these two causes, yet the modified condition is no doubt often due to both. The mammalian mother has two chances to injure an offspring, either by producing a defective egg, or by supplying an unfavorable or diseased environment in which the embryo must develop.

The final combination is the mating of two alcoholic individuals; this, of course, offers the greatest chance for defective offspring.

Effects of Alcohol on Offspring of Treated Animals

Condition of the Animals	Number of Matings	No Result or Early Abortion	Still-Born Litters	Number of Still-Born Young	Living Litters	Young Dying Soon After Birth	Surviving Young
Alcoholic ♂ × Norm. ♀	53	22	7	13	24	20	31
Norm. ♂ × Alcoholic ♀	14	2	3	9	9	9	10
Alcoholic ♂ × Alcoholic ♀	27	14	3	6	10	5	9
SUMMARY	94	38	13	28	43	34	50
Control Norm. ♂ × Norm. ♀	33	0	1	4	32	4	56
Results of Mating Second Generation—Untreated							
2d Gen. × Normal	2	0	0	0	2	0	3
2d Gen. × Alcoholic	2	0	2[1]	5[2]	0	0	0
2d Gen. × 2d Gen.	13	4	0	0	9	4[3]	10

[1] 2^n ♀ × a ♂; 2^n ♂ × a ♀.

[2] 1 Det.

The accompanying table shows the results obtained in 94 matings of the treated animals. The upper line shows the effect of mating alcoholic males with normal females. In 53 such matings 22 gave early abortions or no results, while control matings invariably gave offspring. Seven stillborn litters occurred among the 31 productive matings. Twenty-four living litters were born, consisting of 51 individuals. Twenty of these young animals died within a few days showing various nervous conditions and usually dying in convulsions.

The second line gives similar results when normal males are mated with alcoholic females. The injurious effects shown by this combination are only slightly more marked than in the above case, where the male alone was treated.

The next line indicates the result of the combination between alcoholic males and females. At this stage of the experiment the results shown here are little if any worse than those in the above cases.

A summary of the outcome of the matings of alcoholic individuals may be expressed as follows: out of a total of 94 matings 38, or more than 40 per cent., were negative or resulted in early abortions, the mother often eating the embryos, so that they could not be definitely recorded. Thirty-three control matings never failed to give results. Of the 56 alcoholic matings which gave results 13, or 23 per cent., were stillborn litters while 43 were living litters which consisted of 84 individuals, of these 40 per cent. or 34 individuals died within a few days.

The 33 control matings gave rise to only one stillborn litter, in 32 living litters of 60 individuals only 4 young, or 6 per cent., died. The results of these matings stand in striking contrast to those of the treated animals.

The lower part of the table gives the data obtained from the few combinations which have been made up to the present time with the offspring from the treated animals. These we may term the second generation. The animals themselves have not been treated although they were derived from treated parents.

In 2 cases second generation animals were mated with normal individuals and the results were perfect—two living litters were born consisting of three young. If one may speak from so few cases it would seem as though the normal mates had counteracted any weakness that may have been present in the other animals.

When two second generation guinea pigs were mated with two alcoholic animals the results were almost disastrous. Two stillborn litters were produced, consisting of five fœtuses, one of which was deformed. It must be recalled that in the above section of the table the 27 matings made between animals both of which were alcoholic gave in all only 3 stillborn litters.

Thirteen matings of second generation males and females gave negative results in 4 cases, or about 30 per cent., nine living litters were born consisting of 14 individuals, four died within a few days after birth, one of which was deformed, and ten survived. It will be noticed that two deformed young have occurred in these 17 second generation matings while in the offspring resulting from the 127 matings above no deformity was observed.

One of these deformed specimens was entirely eyeless. This is of interest in connection with the experiments on the fish and bird, described above. It would seem as if this alcohol treatment had produced a weakened race. The embryos developing in such a mother have insufficient developmental vigor to form a perfect nervous system and in the one case the eye anlagen were completely unable to arise or differentiate so that an eyeless individual resulted. The optic nerves and tracts are, of course, absent. This eyeless guinea pig had an alcoholic grandfather, its maternal ancestry consisted entirely of untreated animals.

Random Group of Matings

2n Nov. 4—100♀ × 92♂ = 0.
2n Nov. 4—101♀ × 99♂ = 0.
2n Nov. 15— 91♀ × 93♂ = Feb. 18—Two apparently normal offspring.
2n Nov. 15— 98♀ × 95♂ = 0.
2n Nov. 16— 76♀ × 77♂ = Jan. 28—Three offspring died few days, one deform.
2n♂ Nov. 16— 88♀ × 78♂ = Feb. 20—One normal, long gestation.
2n♂ Nov. 16— 87♀ × 75♂ = Jan. 27—Two small offspring.
♂ Nov. 16— 66♀ × 5♂ = 0.
♂ Nov. 16— 19♀ × 6♂ = Jan. 30—Three, two died at birth, one weakling.
♂ Nov. 16— 34♀ × 43♂ = Feb. 4—Two apparently normal.
♂ Nov. 16— 49♀ × 45♂ = Jan. 31—Three apparently normal.
C Nov. 16— 33♀ × 46♂ = Jan. 30—Two large offspring.
C Nov. 23— 50♀ × 69♂ = Feb. 5—Two large offspring.
C Nov. 23— 15♀ × 70♂ = Feb. 4—Two large offspring.
C Nov. 23— 54♀ × 72♂ = Feb. 3—Two large offspring.
C Nov. 23— 56♀ × 80♂ = Feb. 3—Three large offspring.
C Nov. 23— 52♀ × 81♂ = Feb. 12—Two large offspring.
C Nov. 23— 53♀ × 48♂ = Feb. 3—One very large offspring.

The next table shows a random group of eighteen matings and gives an idea of the individual responses obtained during the experiments which have now been conducted for two and a half years. These represent the last November matings. Four of the 18 gave negative results, one of these was a double alcoholic mating, at this stage of the experiment this mating generally fails to give offspring. The other failures were from the second generation matings. These failures are not due to the fact that the animals are inbred or that they do not continue to breed in the laboratory. Some of the control matings are from second and third generations kept under identical conditions.

It is hoped that these experiments may be further regulated so as to give more definite responses of the nature obtained with the lower forms, fish and birds. At the present they show that such treatments tend to lower developmental vigor and give rise to weak and more or less degenerate types of offspring.

These experiments are the first to show in a definite manner that the mammalian offspring may be injured or modified in its development by treating the male parent so as to affect his germ cells.

For a more detailed account of most of these experiments the reader is referred to *Jour. of Exper. Zool.*, 1906, III, 99; same, 1907, IV, 165; same, 1909, VI, 285; *Arch. f. Entwicklungsmech.*, 1907, XXIII, 249; same, 1912, XXXV, 569; *Am. Jour. Obst.*, 1909, LIX, No. 4; *Am. Jour. Anat.*, 1910, X, 369; *Arch. of Internal Medicine*, 1912, X, 369.

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CONCERNING THE MECHANISM AND DIRECTION OF EMBRYONIC FOLDINGS

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THREE FIGURES

There are three means by which a germ layer having the epithelial type of structure may expand (Minot): by the multiplication of cells; by the enlargement of cells; and by the flattening out of cells.

One or more of these methods, sometimes perhaps combined with other factors, are responsible for the expansion of any form of structure; they do not apply to the epithelial types of germ layers alone, but to all growing tissue. They are general. There is, however, an especially striking resemblance between the surface enlargement of a germ layer and that of the different kinds of epithelia.

The three modes of surface increase are not of the same importance in accomplishing the desired effect, the multiplication of cells is the most essential. There is also, it seems, a marked difference between 'multiplication of cells' and 'enlargement of cells' on the one hand and 'flattening out of cells' on the other. The first two might well be called an active expansion of the surface, while it seems appropriate to regard the third largely as a passive expansion. When, for instance, in the formation of the limb buds the mesoderm increases rapidly, the ectodermal epithelium, as far as the actual folding process is concerned, seems to take little *active* part in the expansion, but is pushed out and stretched in a more or less passive manner. Incidentally, of course, the ectoderm will also continue to increase its surface by multiplication of cells, but this process is not responsible for the formation of the limb bud. In many instances, however, the epithelium increases its mass without being forced by the underlying tissue, and this is true in the formation of most of the important organs of the body. This *active* growth of the epithelium gives rise to the anlagen of the sense organs, lungs, liver, pronephros, mesonephros, and so forth.

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[1] This paper was in part worked out in the Histological Laboratory of the University of Munich. To Professor Mollier, head of the institution, my thanks are due for kindly furnishing me a place in his laboratory.

Wiedersheim states for the formation of the lungs:

> An ihrer Bildung sind beide Blätter, Mesoderm und Entoderm, beteiligt: letzteres aber spielt beim Zustandekommen der gesamten Lungen-Architektur weitaus die Hauptrolle. Das *Entoderm* ist als das *treibende, formative* Princip zu betrachten. Es führt ein central fortschreitender Sprossungs- und Knospungs-Process des entodermalen Epithels zur Vervollkommnung der Lunge. Ontogenie und Phylogenie gehen parallel, und hier wie dort richtet sich die mesodermale Lungenanlage formell nach der entodermalen.

All the most important parts of the body arise from epithelial structures. Epithelium is the first structure to appear in development (blastula wall) and the first differentiation of this epithelium is most commonly gastrulation by invagination. This invagination is essentially the same process as that by which the anlagen of all the organs are derived from epithelium, even after an epithelium has become highly differentiated, and no matter from which germ layer it arose.

There begins a more *active* growth in special parts of an epithelium, which results in an invagination, if the growth occurs in a covering epithelium, while on the other hand it leads to an evagination in a lining epithelium. The ectoderm on the outside of the body invaginates to form the lens, ear, nose, ectodermal gill parts, mouth and so forth, yet as soon as it lines a cavity, it evaginates to form the optic stalk, infundibulum, and so forth. The entoderm does the same in forming the lungs, thyroid, liver and so forth. It thus seems to be the rule that *the epithelium as a result of active growth always bends towards the underlying mesoderm, that is, always away from a free space*, be it a cavity or the outside of the body.

A point of particular significance is the fact, that all of these foldings, either evaginations from the lining epithelia or invaginations from the covering epithelia, are always in the same direction as the original gastrular invagination. They are continuations in the original direction in which the archenteron began to fold, and all subsequent folds of the entoderm are still further inward, as liver, lungs, and so forth; while the ectoderm remaining outside always invaginates to form organs, just as the blastula invaginated to produce the gastrula.

The visible signs of a beginning embryonic fold are rather simple. First at the place where the fold will form an accelerated cell multiplication with an increase in the thickness of the epithelium is noticeable. This localized increase must necessarily lead to the formation of a fold, if the other parts of the epithelium or the underlying tissue do not allow of expansion. Yet there are other factors necessary always to turn these folds in the proper direction, that is, towards the underlying mesoderm or towards the space in which mesoderm will later develop. One of these factors might be found in the polarity of the epithelial cells. In any epithelial cell that part by which it is attached to the underlying tissue is more rigid than the free part. Nucleus, granules, and so forth, are usually located in the basal part of the cell. Even in the adult body many kinds of epithelial cells give evidence of polarity (Rabl), which means "a differentiation of protoplasm along the axis of the cell" (F. T. Lewis). The free and basal parts in themselves demonstrate the polarity of the epithelial cell. When cells have lost this specific polarity they no longer form epithelia but make up supporting and other kinds of structures. As an evidence of polarity cilia and flagella appear only on the free surface, while the nutrition of the cell takes place from the other pole.

When a part of an epithelium increases rapidly without being able to expand, the neighboring parts of this epithelium press on the increasing region and force it out of their level. It seems easier to compress the less stiff, free parts of the cells, than the stiffer, basal portions, which difference may cause the fold to start towards the underlying tissue. Hand in hand with the pressure from surrounding portions works that of the growing cells themselves. The increasing mass of the basal poles will tend to relieve the pressure in the direction of the arrows *a–a*, as indicated in figure 1.

In all active folding processes, therefore, the basal poles of the epithelial cells will form the apex of the fold. A purely mechanical principle could be held responsible for this effect.[2] However, it is impossible to say that nothing but mechanical factors are concerned in all the folding processes of the early embryo. I am simply attempting to explain the mechanical side of such processes; for one things is certain, that no folding, or any other process in development, could take place, if the proper physical conditions did not exist.

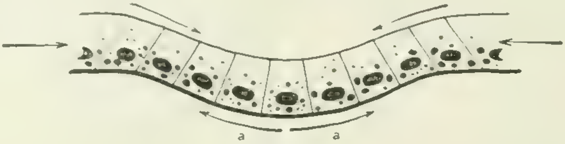

Figure 1

The same mechanism seems to play an important part even in the earliest folding process of the majority of organisms, gastrulation by invagination. A blastula can easily be compared to a single-layered epithelium enclosing a cavity, except that the basal parts of the cells form the lining of this cavity, while in all other cavities later than the blastocoel, the free poles of the cells line the lumen. On account of this condition the blastula is the only stage in which the active folding projects *into* the cavity, in all other cases it starts *away* from the cavity. The original direction of the fold in regard to the topography of the cell remains therefore unchanged.

The blastocoel is not a true cavity as is for instance, the neural tube, the cavity of the lungs, and so forth. It is wanting in some cases (epibolic gastrulation) and very much reduced in others. The first true cavity to appear is the gastrocoel, and the gastrocoel is formed in exactly the same manner as the neurocoel and other cavities.

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[2] The physicist is familiar with this phenomenon of bending. When two strips of paper of different thicknesses are pasted together and caused to bend either by warming or moistening them gradually, the thinner paper will always be on the concave side of the fold.

That the lining parts of the blastula cells are their basal poles, must be concluded from two factors. First, there are many blastulae, which possess cilia on their outer surface. Second, after gastrulation has taken place, the formerly outer poles become the lining surface of the digestive tract, while the formerly inner poles will be attached to the mesoderm as basal poles.

In gastrulation, therefore, the basal poles of the blastomeres form the apex of the folding in exactly the same manner as in every later folding process.

Through gastrulation a part of the blastula wall is brought into the blastocoel to form the entoderm. All later foldings of this entoderm go in the same direction as its first (gastrulation) fold; that is, always deeper inward and away from the cavity. The later folds of the entoderm are, therefore, only branches or ramifications of the first fold.

The ectodermal part of the blastula wall which does not invaginate retains its property to do so. All the later foldings of the ectoderm go in exactly the same direction as the first fold, they too are invaginations.

Ectoderm and entoderm, therefore, seem to be able to fold actively in one direction only, and the entoderm has retained this property from the time when it was still ectoderm.

The question now arises: why does the first folding process occur?

It seems that the above mentioned prerequisite of all folding processes, namely, the localized increase in mass or 'plate formation,' as it is called in the most typical cases, appears also in the invaginating gastrula as the preliminary stage of the folding.[3]

[3] "The egg of Lineus lacteus undergoes equal cleavage, from which a regular blastula results. The latter loses its regular form before invagination begins. The cells at the vegetal pole increase considerably in size" (Metschnikoff); this is true of many other cases. "In Amphioxus the gastrulation begins by a flattening of the vegetative part of the egg" (Morgan and Hazen); and this also is true of other eggs.

If segmentation of the blastomeres should go on beyond the normal point without gastrulation the result would be a continual enlargement of the blastula, provided there was enough material for division present or available for assimilation from the surrounding medium. This, however, is not the normal course of events. After segmentation has been going on for some time the two poles of the blastula usually show differences. The cells at the vegetal pole are larger and higher than those at the animal pole. When the micromeres continue dividing they either press against the macromeres, and cause them to invaginate, or try to grow around them. The latter assumption was first made by Goette. Both processes will be furthered by the simultaneous expansion of the macromeres causing their basal poles to press in the same direction (figs. 2 and 3, in which the position of arrows indicates the direction of pressure).

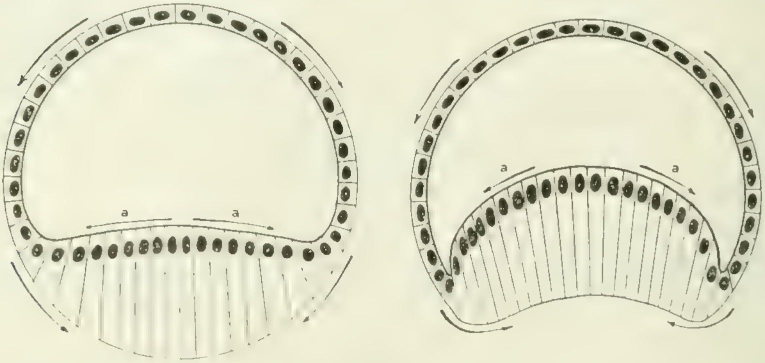

Figure 2

Epibolic gastrulation, as seen in Ctenophores and Annelids, seems to indicate that not only the growth of the macromeres, but also the rapid expansion of the micromeres, are forces concerned in gastrulation. The effect of the excessive growth of the ectoderm is seen most strikingly in the placula of Nematodes, and so forth, as described by Bütschli. Gastrulation in the Ascidiae is also to be kept in mind.

Since in the cases of gastrulation by typical invagination the non-invaginating cells are smaller than those which are to move into the blastocoel, the former are enabled to push over the latter thus assisting in the process leading to invagination. In Herbst's Lithium larvae all the blastomeres were swollen and showed a vacuolar appearance. Shortly before (exo-) gastrulation began, the entoderm cells lost their vacuoles and thus became *smaller* than the ectoderm cells. This is just the reverse of the normal happenings, and since gastrulation took place in the direction opposite to the normal, the surmise is warranted that the size of the blastomeres plays an important part in gastrulation. In the Lithium larvae the larger 'micromeres' were not able to push over the smaller 'macromeres.' The larger cells, on the other hand, did not start to invaginate because the arch formed by them was so much larger than that of the smaller cells (cf. p. 418).

There are, however, a few cases on record in which the cells at the vegetal pole are the smaller ones, yet apparently they invaginate. Most of these cases are met with in the Scyphomedusae. "In spite of the numerous reports on this subject, many of which are contradictory, the process of germ layer formation in this group of animals is not yet perfectly understood" (Korschelt and Heider). Conklin describes gastrulation by unipolar immigration or invagination in different eggs of the same animal, Linerges mercurius, and states "that in other genera of Scyphomedusae all forms of gastrulation may occur." Hyde and Smith make similar statements for Aurelia flavidula and marginalis, and many other authors claim the same for the rest of the Scyphomedusae. Thus Korschelt and Heider were, recently (1910), forced to state "that at present it is impossible to get a clear picture of the formation of the entoderm in this group of animals. Later investigations will have to show, whether or not one or more of the important stages of development have escaped the observations of the authors, (Hyde and Smith)."

One fact seems certain, that the formation of entoderm by true invagination is not the typical case in the Scyphomedusae, therefore, other factors differing from the one discussed here may work in this special mode of gastrulation.

There is, however, a point of interest in this somewhat indeterminate type of gastrulation as compared with the typical gastrulation by invagination. In the latter type the row of micromeres is always longer than that of the macromeres, while in the Scyphomedusae the row of non-invaginating macromeres is longer than that of the cells which are to move towards the blastocoel. There may be some mechanical force connected with this phenomenon. Rhumbler has compared the blastula with a bow, the ectodermal cells forming the arch, the entodermal the cord. In all blastulae the ectodermal 'arch' is longer than the entodermal 'cord,' even if the cells at the vegetal pole are smaller than those on the other. The arch then, according to Rhumbler, would always enact some influence on the cord, and vice versa.

Cases have been described in which there was apparently no difference between the two poles of the blastula. In Terebratulina septentrionalis Conklin "finds it quite impossible to distinguish any difference between the cells which invaginate and those which do not, until after the gastrulation is well advanced. Gastrulation occurs by typical invagination and at the time when the infolding begins, there is no difference in the cells at the two poles."

In such cases, invagination might start at any place since the inner poles of the cells are everywhere under less pressure than the outer poles. Yet the region, where it occurs, must be somewhat different from the neighboring parts, although this difference may not be discernible.

That there are differences in the blastomeres, although not always in size, is stated by Metschnikoff in a description of Aeginopsis: "Although in the 16-cell stage the blastomeres do not differ in size, they show other differences. Some cells reach the surface of the blastula with a large part of their body, others only with a small portion. The latter cells, irregularly distributed over the surface of the embryo, later on get into the blastocoel . . . . " This case seems to indicate that it is simply pressure which pushes some blastomeres into the blastocoel, because before these cells start to migrate they are already somewhat pressed away from the surface.

Roux and Rhumbler were the first to point to the different sizes of the blastomeres as being influential in gastrulation. Rhumbler, however, believes that chemotaxis alone is the inducing factor of gastrulation, while mechanical forces serve merely to assist the process after it has once begun. Yet there are some reasons, at least, to think that mechanical agents alone might force the entodermal cells towards the blastocoel.

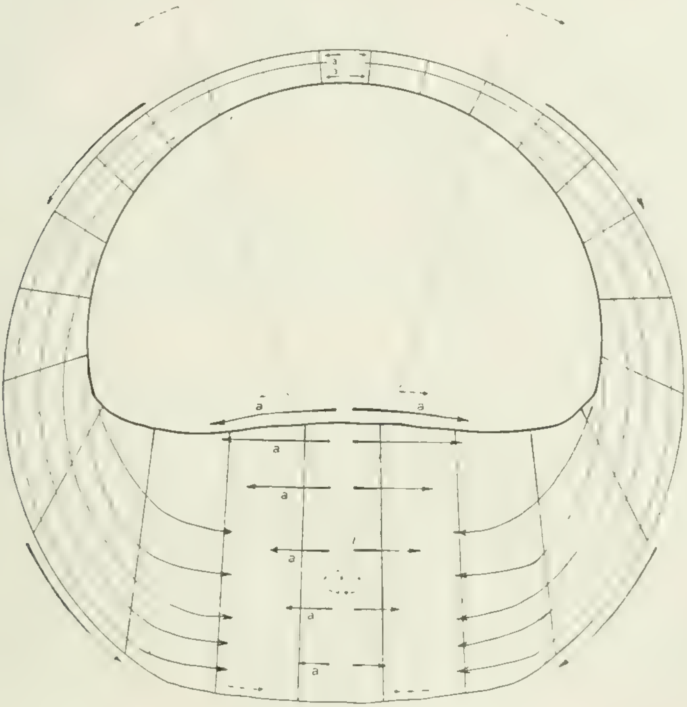

Figure 3

Figure 3, in which the direction of pressure is indicated by the arrows, will show, how mechanical forces might play a very important part in invagination. The compression from the side of the ectoderm will be the greater, the nearer to the periphery the compressed region lies. On the other hand, the tendency to expand will be greatest near the basal poles, since in this region lie more of the formed parts of the cells (yolk granules, etc.).

During segmentation of yolk-laden eggs the yolk becomes located more and more towards the inner parts of the blastomeres. "And it is clear," Assheton says, "that the distribution and quantity of yolk relative to the upper and lower poles is an all important factor in the process of invagination." The relative position of the nuclei of the blastomeres varies in different species. In the literature there are hardly two statements which agree in this respect, while most of the illustrations of gastrulation are so diagrammatic as not to allow of interpretations. However, the location of the nucleus, as a centre of force, may also play an important part in gastrulation, as Assheton recently tried to point out (cf. Assheton).

With each cell division the ectodermal pressure becomes greater. In consequence of this more and more of the formed and liquid content of the entodermal cells will be pressed to their inner poles, which in turn will create a greater tendency on the part of this region to expand, and finally both forces will produce the invagination.

If, in addition the cells take up material from the blastocoel, as Rhumbler states, the increasing pressure on the basal poles will still further this effect.

The diagram 3 also shows, why the micromeres do not invaginate. In them, of course, the pressure differences between basal and peripheral poles are much smaller than in the macromeres.

In Roux's ('97) gastrulation experiments with oil drops, chemotaxis certainly played no part in forcing the larger oil drop into the 'segmentation cavity' (Roux's fig. 21). It was simply pushed in by the pressure of the smaller oil drops.

Rhumbler claims that this phenomenon cannot be compared with actual gastrulation, since dead cells like the oil drops would be forced from the region of high to that of low pressure, while in a row of living cells they all would flatten out to a mere film, if necessary.

This reasoning would be true, if the pressure were on both sides ideally equal along the entire height of the cells, but such is not the case, as the diagram shows. When a piece of skin is pressed between two fingers the cells do not flatten out, but are simply forced from the region of high to that of low pressure. There is, therefore, a certain resemblance between Roux's oil drop experiments and the natural process of pressing the entodermal cells into the blastocoel. The oil drop, however, loses its connection with the neighboring drops. The invaginating cells would do the same, and often do, but for the reason that the blastomeres have a strong hold on one another (Cytarme), while the oil globules have not. In addition, the number of cells under pressure is not limited, those cells which have not yet invaginated are also under pressure and are thus forced to follow the invaginating ones.

Rhumbler's assumption that chemotaxis plays an important part in gastrulation is no doubt partially correct. The mechanical influences, however, are also of importance. If migration of cells caused by chemotaxis was the only factor accomplishing gastrulation, it would be difficult to understand why actively migrating cells keep up a definite epithelial arrangement and do not become disarranged. There is attraction between the cells (Zur Strassen), and there can scarcely be attraction and active migration in equilibrium at a given time.

Active migration of the future entodermal cells might perhaps explain gastrulation by polar and multipolar immigration, the latter being a rather rare type of gastrulation. Yet mechanical influences pushing some cells out of the level of the others might explain it as well (Aeginopsis).

Multipolar immigration is just the fact that seems to argue most against the theory that chemotactic influences are the main forces concerned in gastrulation. Yet many biologists regard these cases as the best proof of chemotaxis. Metschnikoff and others have assumed that some blastomeres actively migrate into the blastocoel on account of certain ('phagocytic') properties they possess and because they are thus destined later on to form entoderm. These cells are distributed irregularly over the blastula surface. Many experiments and observations, however, have shown that in blastulae with a 'determinate cleavage' the cells that are destined to form special parts of the embryo lie in definite groups. The cell groups come, of course, from certain early blastomeres which have transmitted to them their specific potential factors (cell lineage). Therefore, if the cells, which in multipolar immigration move into the blastocoel, would immigrate only for the one reason that they have specific qualities which the remaining cells do not possess, or because they are 'destined' to form entoderm, one should expect to find them grouped in a certain zone of the blastula, as they are descendants of one or more cells of the early cleavage stages. They are, however, irregularly distributed over the surface of the blastula which makes it seem improbable that they possess qualities differing from those of the rest of the cells. They are more probably forced into the blastocoel by outside conditions, and later on, because they are lying within the blastocoel, they undergo such histological differentiation as to make them fit to form entoderm.

It is obvious that chemotaxis with consequent active migration is not always necessary to induce gastrulation. The epibolic type of gastrulation, for instance, cannot be explained by chemotactic migration, since here the macromeres lie within the segmentation cavity, while the micromeres tend to grow over them. If chemotropism plays the important part in invagination as is generally supposed, it is probably only one of the factors creating the proper pressure conditions that are necessary for invagination. It seems, however, that the pressure in the blastula wall alone would suffice to start the invagination, since the inner poles of the blastomeres are always under lower pressure than the outer ones.

By warming sea water to 30°C., Driesch could force Sphaerechinus blastulae to undergo exo-gastrulation, a phenomenon which shows that gastrulation is dependent on physical factors. Driesch states "that the growth of the blastula wall is the vital, fundamental process; the direction towards which the growth proceeds is determined by the surroundings."

In another series of experiments Driesch separated the micromeres from the macromeres of Echinus blastulae by shaking. In both groups the respective cells arranged themselves as blastulae, and in both types of blastulae, after cell division had proceeded for some time, invagination took place. Driesch therefore says that "any cell of the blastula wall may give start to the formation of entoderm." The surmise is warranted that, at least in the blastulae composed only of micromeres which normally had no chemotactic tendency to immigrate, pressure was the chief factor in inducing gastrulation.

It is not the purpose of the foregoing discussion to show that mechanical forces are the only factors concerned in gastrulation by invagination, *but rather to emphasize the great morphological similarity shown by all the folding and budding processes in development.* Since there is such a striking morphological resemblance between gastrulation and the anlage of most of the organs, one is justified in looking for similar producing forces.

In summarizing the main points of the discussion it may be stated:

1. One of the forces causing the fold always to turn in the proper direction is very likely to be found in the pressure differences based on the polarity of the epithelial cell.
2. The thickening of the region, which starts to fold, is a preliminary stage in every folding process. Its consequences are: (a) the polar differences in the epithelial cells become emphasized or exaggerated, since the cells become much higher than the rest of the epithelium; and (b) the rapid cell multiplication calls forth the necessary pressure from the neighboring regions to cause the compressed cells to leave the level of the epithelium.

The preliminary thickening of the region prior to folding seems to be universal, a fact that suggests its great importance. This has been observed by many morphologists, yet it has never received the proper comment from a mechanical standpoint.

A few of the instances in which it has been stated morphologically, may be mentioned here.

In man the epithelium of the Wolffian duct increases, according to His, to double the thickness at the place from which the ureter will bud off. Similar observations have been made by Riede in the sheep, by Schreiner in the rabbit, by Sedgwick and others in birds.

H. Rabl studied the development of the Müllerian duct in Salamandra maculosa and found that an area of cylindrical cells appears in the flattened mesothelium, before the invagination for the Müllerian duct takes place. Brauer describes a similar localized increase in height in the peritoneal epithelium of Hypogeophis which leads to the formation of a fold, the forerunner of the Müllerian duct. Braun and Mihalkowicz describe the same process in reptiles. Keibel and Abraham state that in birds the low mesothelium first becomes cylindrical, then increases the number of layers and finally invaginates. In mammals the same process has been seen by many observers.

In Amphioxus, according to Zarnik, the gonads are laid down as foldings following very noticeable elongation of the epithelial cells.

Maurer describes, in Bufo vulgaris, that part of the epithelium, which by folding is to form the post-branchial body, "as different from the rest of the gill cleft epithelium. It contains very high cylindrical cells and is easily distinguishable from the neighboring region."

One or the other of the principles outlined above seems to be involved in every fold leading to the anlage of an organ.[4] None, however, holds good for the foldings of the fetal amniotic membranes. In the formation of the amnion the basal poles of the cells—'basal' in view of the ectoderm from which the amnion arises—form the apex of the fold.

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[4] One exception is the formation of the optic cup. Here the neuro-ectoderm, pushing off from the central canal as optic vesicles, invaginates to form the cup in such a fashion that the *free* poles, those toward the lumen of the nerve tube, form the *apex* of the fold. No possible explanation can be found for this deviation from the usual course. However, one fact is worth considering. Although in the formed retina the region towards the vitreous humor is still the basal one histologically, yet functionally the region towards the pigment (rods and cones) is the more active one. It seems as if a change in the functional polarity of the retina had taken place, as compared with that of the original neuro-ectoderm. Furthermore in the formation of the optic cup the invaginating part, the latter nervous layer of the retina, is many times higher than the outer pigment layer; a difference in thickness of the two layers so characteristic in invagination.

Yet this folding process is a very different mechanism from the anlage of an organ. There is no localized thickening or plate-formation, but the multiplication of cells is uniform all over the sheet, so that the fold is not forced to start in a certain direction. Besides, the cells are so flat that there is hardly a difference between their two poles, and both poles are free. Furthermore, the somatic layer of mesoderm pushes or grows against the ectoderm in a way somewhat similar to the outpushing of the limb buds. For these reasons this folding may be considered as of the passive type. The allantois, on the other hand, arises from the hind gut in a manner entirely in accord with the principles of foldings I have set forth.

So far only active foldings of a germ layer or epithelium have been discussed. In passive foldings a germ layer can expand chiefly by a flattening out of its cells (third mode).

In the formation of the limb-bud, as mentioned in the introduction, the expansion of the mesodermal structures seems to be the main factor causing the ectoderm to fold out with the free poles of its cells at the apex of the fold.

In Sphaerechinus larvae which were raised in sea water and K salts, Herbst observed a retardation in the formation of the skeleton and arms of the pluteus. He remarks "that the outgrowth of the arms is caused by the stimulus created by the growing skeletal parts on the body wall."

Mollier observed in Lacerta that in the formation of the limb buds the mesoderm is the first tissue to increase in thickness, Bardeen and Lewis describe a similar process in man. Thus the growing mesoderm starts the formation of the limb-bud, secondarily the ectoderm increases somewhat in thickness.

In fishes the formation of the extremities is complicated. First an increase in the thickness of the somatopleura by rapid cell division is noticeable, as shown by Boyer for the teleosts, K. Rabl and Mollier for the selachii (first stage). After this mesodermal ridge has reached a certain thickness the covering ectoderm begins to increase its mass (second stage). Later the ectoderm begins to rise up in form of a longitudinal fold (third stage), into which the mesoderm rapidly expands (fourth stage). The interval between the mesodermal thickening and the rising of the ectoderm is different in different animals. In Ceratodus, for instance, the independent folding of the ectoderm is missing entirely, in Torpedo it takes place along with the mesodermal increase (Ziegler).

Stage 3 of the above quoted mode of limb budding, namely, the independent rising of the ectoderm, is apparently not in accordance with the mechanism this paper is supposed to point out. The independently folding epithelium should fold towards the mesoderm. It does just the opposite. Three reasons seem to be worth considering that may explain this apparent exception to the rule:

1. It has been proven that the mesenchym starts the budding; that is to say, it causes or stimulates the ectoderm to begin to fold away from the underlying tissue.

2. The folding of the ectoderm, does not seem to be absolutely independent of the mesenchym; for example, the absence of such folding in some species and its coincidence with the mesenchymal thickening in others. It seems much more likely that the four stages of budding are not as strictly separated from one another as it appears from the description, but do somewhat overlap as in Torpedo.

3. If the ectoderm really does fold independently, than in this special instance it cannot possibly turn towards the underlying tissue, since the latter expands actively towards the ectoderm.

There can be little, if any, doubt that in the formation of the limb buds the thickening of the ectoderm is merely a secondary process. Several authors emphasize this point. Boyer, for instance, says: "I am convinced from my own observations upon shark embryos that in the latter, as in Fundulus, the earliest step in the development of the pectoral fin is not a modification of the ectoderm, as supposed by Balfour and accepted by Dohrn, but that the beginning must be referred to the proliferation of the mesoderm in the somatopleure, as I have already pointed out for Fundulus." Mollier states: "So much I am able to say, that in Torpedo as well as in Pristiurus, and Mustelus the thickening of the somatopleure was noticeable, while the ectodermal cells did not show the slightest increase in height." Also in the limb formation in Lacerta muralis Mollier speaks of a mesodermal with following ectodermal thickening. So Braus is correct in saying "that the ectodermal ridge is the visible sign of the developmental processes taking place in the underlying mesoderm."

That the ectodermal thickening is merely a reaction to the mesodermal thickening also seems obvious from observations made on Torpedo by K. Rabl. The thickening of the mesoderm with following growth of the ectoderm takes place independently in fore and hind extremities. Later a connection between the two separate anlagen is established, the mesoderm increasing first and being followed by the thickening of the ectoderm. This secondary stage Balfour mistook for the primary one and made it the basis for his "lateral fold hypothesis." The connecting portion later disappears, and this shows that the ectoderm increases, as soon as the mesoderm thickens although no formation of an extremity takes place afterwards.

Though it cannot be proven definitely, it may be strongly suggested that the same mechanism that is involved in the formation of the limbs is manifest in the outpushing of the villi in the intestines, chorion, gill filaments, choroid plexus, and so forth. In all these cases the mesoderm increases on account of the rapidly growing blood vessels, while simultaneously the ecto- or entoderm, either reacting to the stimulus from the mesoderm or more likely independently, increases rapidly by cell multiplication, so that both processes practically go hand in hand. Therefore, since the epithelium in these cases cannot fold or bud towards the supporting tissue, it must do so in the opposite direction.

Maurer believes, that in the formation of the intestinal mucous membrane the actively growing mesoderm undoubtedly plays an important part, yet the investigations so far performed have thrown no definite light on the specific mechanism. Indeed, it will readily be seen that the reports by different authors on the formation of intestinal villi are very contradictory.

Gianelli claims that in the reptiles first an increase in the number of epithelial cells takes place, which leads to the formation of a fold, into which connective tissue grows secondarily.

In birds Seyfert finds that the connective tissue gives the impulse to the formation of villi, starting cone-like elevations which push the epithelium into the lumen of the gut. In man and mammals, as Brand and Barth report, epithelium and connective tissue increase simultaneously. Voigt studied this process especially in the pig. Yet Patzelt, on the other hand, claims that the villi are started as epithelial elevations while the connective tissue plays merely a secondary rôle.

More conformity seems to underlie the different reports that have been made on the formation of intestinal glands, Lieberkühn's crypts, and so forth. All authors agree that the glands start as foldings or sproutings of the epithelium towards the mesoderm. This is in accordance with my view that an independent growth of the epithelium will fold towards the underlying tissue. Some discrepancies, however, exist as regards the early anlage of the glands. Oppel speaks of the formation of solid epithelial processes, Voigt, however, observes foldings of the epithelium. Seyfert, in birds, and Gianelli, in reptiles, describe solid, while Barth and Brand, in mammals, speak of hollow outpushings of the epithelium.

In every case, whether a solid or a hollow folding of the epithelium is formed, it always starts towards the mesoderm, and leads to the formation of a gland or crypt, while on the other hand, when the mesoderm grows rapidly a villus or intestinal fold is formed. Thus it seems as if the two opposing theories, Edinger's and Oppel's, may both be accepted as correct in part. Edinger believes in the growth of the mesoderm as initiative and secondarily the entoderm folds leading to the different formations in the intestinal tract, while Oppel holds the entoderm alone responsible. Edinger's view seems to be correct as regards the folds and villi, and Oppel's as regards the glands.

The interaction between the ectoderm and mesoderm is very strikingly seen in the formation of the enamel organ and dental papillae of the developing tooth. The ectoderm at first thickens and starts towards the mesoderm to form the enamel organ and later a cone of mesoderm grows up to form the dental papilla and pushes the ectoderm again towards the outside. Here then both processes are illustrated, the active folding of the ectoderm towards the mesoderm and the passively outpushed ectoderm by the growing mesoderm below. The developing hair undergoes a similar process.

SUMMARY

From all histological observations that have been made on folding germ and epithelial layers, and from a mechanical interpretation of these structures, the following conclusions may be reached:

1. In all folding processes in which an epithelium takes an *active part*, the basal poles of its cells form the apex of the fold.
2. All folding processes in which the free poles of the epithelial cells form the apex, are *passive* folds, caused by the rapid growth of the underlying tissue.

BIBLIOGRAPHY

ABRAHAM, K. 1901 Beitrage zur Entwicklungsgeschichte des Wellensittichs. Anat. Hefte, p. 591.

ASSHETON, R. 1910 The geometrical relation of the nuclei in an invaginating gastrula (e.g., Amphioxus) considered in connection with cell rhythm, and Driesch's conception of entelechy. Arch. f. Entw., Bd. 24, p. 46.

BARDEEN, C. R. AND LEWIS, W. H. 1901 Development of the limbs, bodywall and back in man. Amer. Jour. Anat., vol. 1.

BOYER, 1892 The mesoderm in teleosts, especially its share in the formation of the pectoral fin. Bull. Mus. Comp. Zool., Harvard, 23.

BRAUER, A. 1902 Beiträge zur Kenntnis der Entwicklung und Anatomie der Exkretionsorgane. Zool. Jahrb., An. Abt., Bd. 16, p. 1.

BRAUN, M. 1878 Das Urogenitalsystem der einheimischen Reptilien, entwicklungsgeschichtlich und anatomisch bearbeitet. Arbeit. d. zool. zoot. Inst., Würzburg, Bd. 4.

BRAUS, H. 1906 Die Entwicklung der Form der Extremitaten und des Extremitätenskeletts. Hertwig, Handb. d. Entwickl., Bd. 3, p. 2.

BÜTSCHLI, O. 1883 Bemerkungen zur Gastraeatheorie. Morphol. Jahrb., Bd. 9, p. 415.

CERFONTAINE, P. 1906 Recherches sur le développement de l'Amphioxus. Arch. Biol., tome 22.

CONKLIN, E. G. 1909 The habits and early development of Linerges mercurius. Publ. Carnegie Inst., Washington, 103.

1892 The embryology of a Brachiopod. Proc. Amer. Phil. Soc., vol. 41.

DRIESCH, H. 1893 Exogastrula und Anenteria. Mitteil. Zool. Stat. Neapel, Bd. 11, p. 221.

1894 Analytische Theorie der organischen Entwicklung. Leipzig.

GIANELLI, Struttura ed istogenesi dell' intestino digestive nella Seps chalcides. Atti R. Ac. Fisiocritici, Siena, ser. 4, tomo 12, p. 11.

GOETTE, A. 1884 86 Abhandlungen zur Entwicklungsgeschichte der Thiere, Heft 2–3, Hamburg–Leipzig.

HERBST, C. 1896 Über das Ineinandergreifen von normaler Gastrulation und Lithium-Entwicklung. Arch. f. Entw. Mech., Bd. 2, p. 455.

HERTWIG, O. u. R. 1879–83 Studien zur Blättertheorie. Heft 1–5, Jena.

HIS, W. 1868 Untersuchungen uber die erste Anlage des Wirbeltierleibes. Leipzig.

1874 Unsere Körperform und das physiologische Problem ihrer Entstehung. Leipzig.

1894 Über mechanische Grundvorgänge tierischer Formenbildung. Arch. f. Anat. u. Physiol. Anat. Abt.

HYDE, J. H. 1894 Entwicklungsgeschichte einiger Scyphomedusen. Ztschr. wiss. Zool., Bd. 58.

KEIBEL, F., U. ABRAHAM, K. 1900 Normentafeln zur Entwicklungsgeschichte des Huhnes. Jena.

LEWIS, W. H. 1902 The development of the arm in man. Am. Jour. Anat. vol. 1.

MAURER, F. 1888 Schilddrüse, Thymus und Kiemenreste bei Amphibien. Morphol. Jahrb., Bd. 13.

1906 Die Enwicklung des Darmsystems. Hertwig, Handb. d. Entwickl., Bd. 2, p. 1.

MAYER, P. 1886 Die unpaaren Flossen der Selachier. Mitteil. Zool. Stat. Neapel.

METSCHNIKOFF, E. 1882 Über die Gastrula einiger Metazoen. Ztschr. wiss. Zool., Bd. 37.

VON MIHALKOWICZ, 1885 Untersuchungen über die Entwicklung des Harn und Geschlechtsapparates der Amnioten. Intern. Monatsschr. f. Anat. u. Physiol., Bd. 2.

Minot, C. S. 1909 Laboratory text-book of embryology; 2nd edition.

Mollier, S. 1895 Die paarigen Extremitäten der Wirbeltiere. Anat. Hefte, Bd. 5.

Morgan, T. H., and Hazen, A. H. 1900 The gastrulation of Amphioxus. Jour. Morph., vol. 16, p. 569.

Oppel, A. 1896, 1900 Verdauungskannal. Merkel-Bonnet, Erg. d. Entwickl.

Patzelt, 1882 Über die Entwicklung der Dickdarmschleimhaut. Sitzber. Ak. Wiss. Wien.

Rabl, C. 1889 Über die Principien der Histologie. Verh. Anat. Ges., Berlin, Bd. 3, p. 39.

1893 Theorie des Mesoderms. Morph. Jahrb., Bd. 29.

Rabl, H. 1904 Über die Entwicklung des Tubentrichters und seine Beziehung zum Bauchfell bei Salamandra maculosa. Arch. f. Mikr. Anat., Bd. 64.

Rauber, 1880 Formbildung und Cellularmechanik. Morph. Jahrb., Bd. 6.

Riede, 1887 Untersuchungen zur Entwicklung der bleibenden Niere. Inaug. Diss. München.

Roux, W. 1896 Über die Bedeutung 'geringer' Verschiedenheiten der relativen Grösse der Furchungszellen für den Charakter des Furchungsschemas nebst Erörterungen über die nächsten Ursachen der Anordnung und Gestalt der ersten Furchungszellen. Arch. f. Entw. Mech., Bd. 4, p. 1.

Rhumbler, L. 1902 Zur Mechanik des Gastrulationsvorganges, insbesondere der Invagination. Arch. f. Entw. Mech., Bd. 14, p. 401.

Schreiner, K. E. 1902 Über die Entwicklung der Amniotenniere. Ztschr. wiss. Zool., Bd. 71, p. 1.

Sedgwick, A. 1880 Development of the kidney in the relation with the Wolffian body in chick. Quart. J. Micr. Sc., vol. 20, p. 146.

Seyfert, 1897 Beitrage zur mikroskopischen Anatomie und zur Entwicklungsgeschichte der blinden Anhänge des Darmkanals bei Kaninchen, Taube und Sperling. Inaug. Diss., Leipzig.

Smith, F. 1891 The gastrulation of Aurelia flavidula. Bull. Mus., Harvard, vol. 22.

Stockard, C. R. 1907 The embryonic history of the lens in Bdellostoma Stouti in relation to recent experiments. Am. Jour. Anat., vol. 6, p. 511.

1910 The independent origin and development of the crystalline lens. Am. Jour. Anat., vol. 10, p. 393.

Voigt, J. 1898 Zur Entwicklung der Darmschleimhaut. Nachr. Ges. Wiss., Göttingen, mat.-naturw. Kl., Bd. 4, p. 416.

Zarnik, B. 1904 Über die Geschlechtsorgane des Amphioxus. Zool. Jahrb., An. Abt., Bd. 21, p. 253.

Ziegler, H. E. 1888 Der Ursprung der mesenchymatischen Gewebe bei den Selachiern. Arch. f. mikr. Anat., Bd. 32.

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FEEDING EXPERIMENTS ON TADPOLES

II. A FURTHER CONTRIBUTION TO THE KNOWLEDGE OF ORGANS WITH INTERNAL SECRETION

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TWO DOUBLE PLATES

Certain mammalian glands with a so-called internal secretion, when given as food, can enact a decided influence on the growth and differentiation of amphibian embryos. This was shown by experiments that were carried out during the summer of 1911 on tadpoles of Rana temporaria and Rana esculenta.

During the spring of 1912 these experiments were repeated and at the same time varied to such an extent that no doubt as to their results remained. Although the experiments of the two seasons revealed many precise data, there is still a great number of obscure features regarding the definite action of internally secreting glands when fed to the tadpoles. While the influence on growth and differentiation resulting from feeding some of the glands was striking, others exerted no marked effects. The action of the latter, in part or entirely, may not be concerned with those most important physiological processes in the embryo, namely, growth and differentiation. They might play their chief rôle, then, in the household of the postembryonic organism. Or, since taken from mammals, some of these internally secreting glands, if at all connected with embryonic development, may fail to reveal this influence, when fed to amphibian embryos. Their action must finally be studied in experiments on higher vertebrates.

The experiments in 1912 were performed on tadpoles of Rana temporaria, Rana esculenta, Bufo vulgaris and Triton alpestris.

The eggs of these species were collected from ponds in the vicinity of Munich and reared in the laboratory. They were kept in large aquaria and after hatching were transferred to smaller dishes, each dish containing about 100 individuals. After the larvae had grown for some time and seemed to be crowded in the dishes they were separated into smaller groups. To avoid possible errors, a great number of individuals was always used for each kind of experiment. All in all, over 20.000 tadpoles were treated.

The eggs from different localities were kept separate and, as far as possible, eggs apparently coming from the same mother were grouped in one set and used for a special experiment. Whenever the eggs necessary for one experiment could not all be supplied by a single female I endeavored to select eggs coming from the same locality, and apparently in the same stages of development. For example, set III probably contained eggs from three mothers, but the time of hatching, April 7 and 8, and their sizes, 10 to 11.5 mm at the beginning of the experiment, show them to be in similar stages of development. Again, for other experiments in which a large number of animals was required, as in sets VIII to XI over 4000, several sets in apparently the same stage of development were thoroughly mixed and then divided into smaller groups. In these last sets the tadpoles hatched between April 10 and 13 and a month later, when the feeding began, their sizes varied from 13 to 18 mm; this difference is not greater than one would encounter in eggs from a single individual.

The water was changed at least once a day. While standing, its temperature varied with the room temperature, a process that corresponds to natural conditions. However, even in a room with apparently uniform temperature, the water will not show exactly the same temperature in all the dishes, as Barfurth has pointed out. To overcome any unequal influence of light, air and temperature resulting from the position of the dishes, they were shifted several times a day so as to progress in a certain order. With this precaution there were no detectable differences of temperature in the dishes assigned to one set. There was sometimes a slight difference of temperature between the dishes used for the different experiments, but this was of no importance, so long as the tadpoles used for one experiment were kept under uniform conditions.

As during the previous year, the food in small pieces was placed in the water and there voraciously taken by the animals, with the exception of pancreas which never seemed to excite their appetite very greatly.

Last year the tadpoles had their natural food previous to the experiments, this year in all but one experiment no other food was received except the one chosen for the studies. In 1911 after the feeding began the animals were kept exclusively on a one-gland diet; in 1912 these experiments were repeated with part of the animals while in addition a mixed, either two-animal tissues or gland-plant diet, was introduced.

The following were used as foods: thyroid, adrenal, liver, spleen, hypophysis, brain, pancreas and muscle from the horse, testicle, ovary and thymus from cattle, and as vegetable food Elodea canadensis and Ceratophyllum demersum.

It would not have been possible to perform such an extensive series of experiments but for the courtesy and generosity of Prof. S. Mollier, in whose laboratory in Munich the experimental part of this paper was carried out. It is with pleasure that I express my best thanks for the many kindnesses shown me during my stay in his laboratory.

Experiment I

Rana temporaria, Set I. Figure 1, *a* to *zf*. Hatched April 4 to 7, 1912. Feeding began April 13, 1912. Original size 10.2 to 12 mm.

The diary reads as follows:

April 4	The feeding begins.
April 22	Thyroid-fed have hind legs forming.
April 24	Thyroid-fed have fore legs forming.
April 25	Thyroid-fed begin to die off.
April 29	Last thyroid-fed animals dead. Thymus- liver- spleen- and muscle-fed tadpoles are larger than the other groups.
April 30	Muscle, liver and spleen ones show hind leg buds.
May 4	Adrenal cortex, adrenal medulla and ovary have hind leg buds faintly noticeable.

TABLE 1

Measurements in millimeters during the experiment

DATE	CONTROL	MUSCLE	MUSCLE-THYROID	LIVER	LIVER-THYROID	SPLEEN	SPLEEN-THYROID	BRAIN	BRAIN-THYROID	ADRENAL CORTEX	ADRENAL CORTEX-THYROID	ADRENAL MEDULLA	ADRENAL MEDULLA-THYROID	OVARY	OVARY-THYROID	THYMUS	THYROID
April 13....								10.5-12.0 mm.									
April 29	12.0-15.0	17.0-21.0		18.0-26.0		18.0-21.0				11.0-20.0		11.0-18.0		14.0-19.0		18.0-24.0	10.0-11.0
May 8	14.0-18.0	18.0-25.0		20.0-29.0		19.0-28.0				14.0-23.0		14.0-22.0		14.0-21.0		19.0-25.0	Dead Apr. 29
	5.0-6.0	7.0-9.0		7.0-11.0		7.0-9.0				4.0-8.0		5.0-8.0		5.0-8.0		7.0-9.0	Completely metamorphosed
	9.0-12.0	11.0-16.0		15.0-18.0		12.0-17.0				10.0-15.0		9.0-14.0		9.0-13.0		11.0-16.0	
	3.0-4.0	4.0-6.0		5.0-6.0		4.0-5.0				3.0-5.0		3.0-5.0		3.0-5.0		4.0-5.0	
May 15..	11.0-19.0	18.0-27.0		20.0-30.0		19.0-30.0		Feeding started May 10		15.0-25.0		15.0-25.0		15.0-21.0		20.0-28.0	
	5.0-7.0	7.0-10.0		8.0-11.0		7.0-10.0				5.0-9.0		5.0-10.0		6.0-9.0		7.0-10.0	
	9.0-13.0	11.0-17.0		14.0-19.0		12.0-20.0				10.0-16.0		10.0-15.0		9.0-16.0		12.0-18.0	
	3.0-4.0	4.0-6.5		5.0-7.0		4.0-6.0				3.0-5.0		3.5-5.0		3.0-5.5		4.0-5.5	
	From now on fed on algae																
May 24	15.0-24.0	19.0-31.0		20.0-32.0		19.0-31.0		16.0-24.0		15.0-28.0		15.0-28.0		16.0-28.0		21.0-31.0	
	5.0-9.0	7.0-11.0		7.5-11.0		7.0-11.0		5.0-9.5		5.0-10.0		5.0-10.5		6.0-10.0		7.5-10.0	
	9.5-16.0	12.0-20.0		14.0-20.0		12.5-20.0		10.0-16.0		10.0-19.0		9.0-17.5		10.0-18.0		13.0-21.0	
	3.0-5.0	4.0-7.0		5.0-7.0		4.0-6.0		3.0-5.5		3.0-6.0		3.5-6.0		3.5-6.0		4.5-6.0	
May 31...	15.5-25.0	19.0-32.0		21.0-32.0		19.0-32.0		16.0-26.5		15.5-29.5		15.0-28.5		16.0-28.5		21.0-31.5	
	5.0-9.5	7.0-11.5		7.5-11.5		7.0-11.0		5.0-9.5		5.5-10.5		5.0-10.5		6.0-10.0		7.5-10.5	
	9.5-16.0	12.0-20.5		14.0-20.5		12.5-21.5		10.0-17.5		10.0-19.0		9.0-18.0		10.0-18.5		13.0-21.5	
	3.0-5.5	4.0-7.0		5.0-7.0		4.0-6.5		3.0-5.5		3.0-6.5		3.5-6.0		4.0-6.5		4.5-6.0	
June 11...	16.0-26.0	19.5-33.0		21.5-33.0		19.0-33.0		16.0-29.0		16.0-31.0		16.0-29.5		16.0-29.5		21.0-32.0	
	5.5-10.0	7.0-11.5		7.5-11.5		7.0-11.5		5.0-10.0		6.0-11.0		5.5-11.0		6.0-10.0		7.5-11.0	
	10.0-16.0	14.0-21.5		14.0-20.5		12.5-22.0		10.0-19.0		10.0-20.5		10.0-19.0		10.0-19.5		13.0-21.5	
	3.0-5.5	4.0-7.0		5.0-7.0		4.5-7.0		3.0-5.5		4.0-7.0		3.5-6.0		4.0-6.5		4.5-6.5	
June 24	16.0-27.5	20.5-37.0	18.5-28.0	21.5-35.0	18.0-28.0	19.0-33.0	11.0-24.0	16.0-32.0	15.0-24.5	16.0-32.5	13.0-21.0	16.0-32.5	15.0-26.0	16.0-31.5	16.0-23.0	22.0-32.5	
	5.5-10.5	7.5-12.5	6.5-9.0	8.0-12.0	6.5-9.0	7.0-11.5	4.0-8.0	6.0-11.5	5.0-7.0	6.0-12.0	5.0-8.5	6.0-11.5	5.5-9.0	6.0-11.0	5.5-7.5	8.5-11.5	
	10.0-17.0	15.0-25.0	12.0-19.0	15.0-23.0	11.5-19.0	12.5-22.0	7.0-16.0	10.0-20.5	10.0-17.5	10.0-21.5	8.0-17.5	10.0-21.0	8.5-17.0	10.0-20.5	10.0-16.5	13.5-21.5	
	3.5-6.0	4.5-7.5	3.5-6.0	4.5-7.0	4.0-5.5	4.5-7.0	3.5-4.5	3.0-6.5	3.0-5.0	4.0-7.5	3.5-4.5	4.0-7.0	4.5-5.0	4.0-7.0	4.0-5.0	5.0-7.0	
July 6....			16.0-19.0		15.0-18.0		9.0-17.0		10.0-17.0		9.5-16.0		10.0-17.5		10.0-17.0		
			6.0-8.0		5.5-8.0		4.5-7.0		4.0-6.5		4.0-6.0		4.5-6.5		4.5-6.0		
			9.0-11.0		8.0-10.0		4.0-10.0		6.0-11.0		5.5-10.0		5.5-11.0		6.0-11.0		
			2.5-4.0		3.0-4.0		2.5-4.0		2.5-4.0		2.5-3.5		3.0-4.0		2.5-4.0		
July 11....	18.0-29.0	24.0-38.0		22.0-32.0		21.0-33.5		19.0-33.0		19.0-33.0		19.0-32.5		22.0-34.0		22.0-33.0	
	7.0-11.0	9.0-11.5		8.0-11.5		7.0-11.5		6.5-12.0		7.0-13.0		7.0-11.5		8.5-11.5		8.5-11.5	
	11.0-18.0	15.0-26.5		15.0-23.0		14.0-22.0		11.5-21.0		12.0-20.0		12.0-21.0		13.5-23.5		13.5-22.0	
	4.0-6.5	4.5-7.5		4.5-7.0		4.5-7.0		4.0-7.5		4.0-8.0		4.0-7.0		5.0-7.0		5.0-7.5	

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[1] When four measurements are given, the first refers to the entire length, second to length of body, third to length of tail, fourth to breadth of body.

May 14	To this time the controls have been starving, in order to test the possible influence of hunger on development. They show no signs of advancement, but are far behind in size. From now on they are fed on Elodea canadensis. A group originally intended for a hypophysis diet also starved up to this day, and from now on is fed on brain. The liver-fed ones have a greenish color, the thymus-fed animals are deep black.
May 24	The smallest individuals in all sets begin to die off. The spleen-fed ones become very black, the bigger ones on adrenal cortex become very light, lighter than on adrenal medulla.
June 12	The first liver-fed ones show hind legs; this is 49 days later than the time at which the thyroid-fed ones attained the same condition.
June 14	Hind legs appear in adrenal-cortex-fed tadpoles. Their body assumes a peculiar triangular shape (fig. 7 *b*), very pointed at the anterior end and broad at the posterior. They are all becoming light, while all the adrenal-medulla-fed ones are dark.
June 17	A number of each group, 70 to 80, which have no hind legs except the small buds, are placed on thyroid diet.
June 19	The light color of the adrenal-thyroid tadpoles is disappearing.
June 20	Those liver-fed ones that have put out fore legs since June 12 shorten their tails. The liver-thyroid animals develop hind legs.
June 21	Adrenal medulla-thyroid and muscle-thyroid tadpoles develop hind legs. The former are extremely active.
June 22	Liver-thyroid and muscle-thyroid ones assume a frog shape.
June 23	Spleen-thyroid and adrenal cortex-thyroid ones develop hind legs. The first liver-fed frog leaves the water.
June 24	Spleen-thyroid and adrenal cortex-thyroid animals assume frog shape. Brain-thyroid ones develop hind legs. Adrenal cortex tadpoles are extremely light greenish-yellow. Brain-fed ones assume somewhat the triangular adrenal cortex shape described on June 14.
June 25	Liver-thyroid and adrenal medulla-thyroid animals show fore legs.
June 26	Spleen-thyroid-fed ones show fore legs. Ovary-thyroid and brain-thyroid tadpoles assume frog shape, the latter are very active.
June 27	Muscle-thyroid animals show fore legs.
June 29	Adrenal cortex-thyroid and brain-thyroid ones show fore legs.
June 30	Ovary-thyroid ones show fore legs.
July 6	All the thyroid-fed ones are fixed, since many of them die.
July 7	Brain-fed tadpoles show hind leg buds.

July 11 Muscle-fed ones have the best developed hind legs, next the ovary and adrenal-cortex ones which have short but very strong legs, then adrenal-medulla, brain and spleen.

The color of the animals on the different diets is: control, brown; muscle, dark brown; liver, dark greenish; spleen, dark; adrenal cortex, very light brown; adrenal medulla, dark brown; ovary, yellowish; thymus, dark; brain, brown.

July 19 Muscle-fed ones begin metamorphosing.

July 20 Ovary-fed ones begin metamorphosing.

Thymus-fed have no signs of legs, spleen-fed only small buds.

In this experiment the thyroid treatment proved to be as effective as in the experiments performed in 1911. Tadpoles that had been fed on no other food than thyroid grew hind legs 9 days after the feeding began and fore legs only 2 days later. Normally several weeks would have elapsed between the appearance of the hind and the fore extremities. When these thyroid-fed tadpoles put out their anterior limbs and began to shorten their tail, they were 18 to 20 days old, calculating from the date of hatching. Normally they would take from 10 to 12 weeks to complete their metamorphosis.

The tadpoles fed on ordinary meat, muscle (fig. 1 *d*, 1 *n*), showed distinct hind legs 44 days and fore legs 71 days later than the thyroid-fed ones. The first individual in the muscle-fed group to complete its metamorphosis was approximately 104 days old.

Liver-fed tadpoles (fig. 1 *a*, 1 *i*, 1 *x*) show the best growth, although their growth is only slightly better than that of the spleen and thymus sets. Next to the thyroid group the liver-fed tadpoles also showed most rapid progress in differentiation. They grew hind legs 40 days and fore legs 49 days later than the thyroid group. The first liver-fed specimen to complete its metamorphosis was approximately 78 days old.

The spleen- (fig. 1 *b*, 1 *l*) and thymus- (fig. 1 *c*) fed tadpoles show almost parallel courses in growth and differentiation. During the first weeks of the experiments the spleen group ran a little ahead of the thymus specimens. The members of both groups became extremely dark in color during the course of the feedings. At the approximate age of 104 days, when the experiment was discontinued, none in either group had hind legs out, although spleen-fed ones showed leg buds.

The three groups of tadpoles fed on adrenal cortex (fig. 1 *f*, 1 *r*), adrenal medulla (fig. 1 *g*, 1 *t*) and ovary (fig. 1 *e*, 1 *p*) were during the first weeks of growth behind the muscle, liver, spleen and thymus groups. Later, however, they grew more rapidly. Some of those fed on ovary actually reached the average size of the faster groups and completed their metamorphosis at the approximate age of 105 days.

The adrenal cortex tadpoles were somewhat faster in development and grew better than the adrenal medulla ones. The former budded hind legs on the 71st and the latter on the 81st day. At the conclusion of the experiment the adrenal cortex ones had strong well developed legs, while the legs of the medulla-fed individuals were still short and drawn close to the body.

In previous experiments, in which adrenal cortex and medulla had not been given separately, it was seen that the adrenal-fed tadpoles became extremely light in color after 3 or 4 weeks of feeding. The pigment cells were found to be completely contracted. The suggestion was made "that the extract from the chromaffine cells of the medulla which dissolved in the water caused the pigment cells to contract" and "former experiments with adrenalin would warrant such a suggestion." In the present experiments, however, separate sets of tadpoles were fed on cortex and medulla respectively. After 5 weeks' feeding those fed on adrenal cortex became much lighter than those fed on adrenal medulla or any other food. This difference in color became more evident as the experiment proceeded, until the cortex-fed tadpoles had an extremely light, greenish-yellow tint. Thus the above suggestion is doubtless incorrect, at least in these experiments, yet the true cause of the light pigmentation is still obscure.

These cortex-fed tadpoles also differed from the other groups in still another feature, namely, the peculiar triangular shape (fig. 7 *b*) of their bodies described above. A few of the brain-fed tadpoles somewhat approached this condition.

The chief purpose of feeding the set I on different foods was to study the influence of the thyroid treatment, after the animals had fed for sometime on various other tissues. The tadpoles were about 10 weeks old, when the thyroid was given. They were fed only four times. This feeding caused the same acceleration in differentiation that was noticed in former experiments. Within 3 to 7 days the reaction to the treatment became evident.

There were differences in the rapidity of this reaction among the different sets of tadpoles, as may be seen from the diary, but whether or not these differences are significant as to the value of the food given before the thyroid treatment, remains an open question.

The liver-thyroid-fed tadpoles (fig. 1 *k*) were the first ones to develop hind and fore legs. This fact might easily be explained on the ground that the liver-fed group was the most advanced in the entire series (May 8, June 1, June 12, and June 23, in the diary). Yet why those fed, for instance, on spleen, the slowest lot in the series, having no legs even on July 19, and those fed on adrenal medulla which were at the start of the thyroid treatment smaller than the liver-fed ones, should react to the thyroid stimulus as fast as the liver group, cannot be explained on the above basis suggested for the liver-fed group.

On the other hand, the adrenal cortex group though of about the same size are 10 days ahead of the adrenal medulla group in differentiation. Yet after thyroid application they do not react with as great speed as the latter, and fall behind in the series.

Throughout the experiment it was observed that muscle-liver- and spleen-fed tadpoles belonged to one class as far as their growth was concerned, while those fed on adrenal cortex, adrenal medulla and ovary formed another class. As soon, however, as the thyroid factor was introduced this classification was disturbed. Thus the previous feeding may influence to some degree the mode of reaction to the thyroid stimulus. No variety of previous feeding can, however, completely prevent this reaction for any length of time. After a shorter or longer interval (in this experiment the difference was only from 3 to 7 days) every tadpole, as far as macroscopic features are concerned, will respond to the thyroid stimulus, no matter what kind of food had been previously given.

To show the different degrees of response to the thyroid food in the various groups, table 2 may be added. While it does not reveal, as said before, any important points as to the respective value of the different foods given before the thyroid diet, it shows some striking changes in the subsequent positions in the series of the various groups, especially those fed on adrenal medulla, spleen and adrenal cortex.

TABLE 2

AVERAGE SIZE AT THE START OF THE THYROID DIET	AVERAGE SIZE 7 DAYS LATER	PERCENTAGE LOSS IN LENGTH		TIME OF REACTION
muscle	muscle	adr. med,	12.761	liver (fig. 1 *k*)
liver	liver	liver,	14.777	adr. med. (fig. 1 *u*, 1 *d*
spleen	adrenal med.	brain,	15.053	muscle (fig. 1 *o*, 1 *za*)
adrenal cortex	brain	muscle,	15.454	spleen (fig. 1 *m*, 1 *z*)
adrenal medulla	ovary	ovary,	16.129	adr. cor. (fig. 1 *s*, 1 *zc*)
brain	spleen	adr. cor.,	28.421	brain (fig. 1 *w*, 1 *ze*)
ovary	adrenal cortex	spleen,	32.692	ovary (fig. 1 *q*, 1 *zb*)

Experiment II

Rana temporaria, Set IV. Figures 4, *a* to *p*. The tadpoles hatched on April 5 to 6, and the feedings began April 13, 1912. Original length of the specimens was 12 to 12.5 mm.

The diary records of the experiment are given below:

April 13 The feeding was started.

April 16 Those fed on thymus and spleen have become larger than the others.

April 17 Thyroid-fed tadpoles have become considerably smaller than thymus-fed ones and the thymus ones are getting dark.

April 18 Thymus-fed tadpoles are much darker than the others, spleen-fed specimens are also getting dark. Thyroid ones now show frog-like bodies.

April 19 Thymus-fed individuals are almost black, spleen-fed very dark, muscle-fed much lighter, thyroid-fed darker than muscle, but lighter than thymus and spleen. Thyroid ones have distinct frog shape, their hind legs are beginning to bud (14 days old).

One set of thymus-fed individuals is now changed to a thyroid diet, thymus-thyroid I.

April 20 The resorption of the tail in thyroid-fed individuals is distinctly noticeable. The specimens all lie on the bottom of the dish and appear frail, taking no more food, while the others in the experiment swim actively.

TABLE 3

Measurements in millimeters during the experiment

DATE	CONTROL	MUSCLE	MUSCLE-THYROID	SPLEEN	SPLEEN-THYROID	THYMUS	THYMUS-THYROID I	II	III	IV	V	VI	THYROID
Apr. 13							12-12.5 mm.						
Apr. 17		14.0		15.0		15.0							13.0
Apr. 20	length												
	14.0-15.0	18.0-19.0		18.0-20.0		18.0-20.0	18.0-20.0						12.5-13.0
	breadth												
	3.0	4.5-5.0		4.5-5.0		4.0-5.0	4.0-5.0						2.5-3.0
Apr. 22							14.0-17.0						12.0-12.5
							4.0						2.0-3.0
Apr. 29	11.0-15.0	18.0-20.0		18.0-21.0		18.0-21.0	8.0-10.0						10.0-12.0
	3.0	4.0-5.0		4.0-5.0		4.0-5.0	3.0						2.0-3.0
May 6	14.0-15[1]	18.0-21.0		18.0-22.0		18.0-23.0		18.0-23.0					
	5.0-6.0	6.0-8.0		6.0-9.0		6.0-9.0		6.0-9.0					
	9.0	11.0-13.0		12.0-14.0		13.0-15.0		13.0-15.0					
	3.0-3.5	4.0-5.0		4.0-5.0		4.0-5.0		4.0-5.0					
May 13	14.0-15.0	18.0-25.0		18.0-23.0		18.0-24.0		10.0-15.0	18.0-24.0				
	5.0-6.0	6.0-10.0		6.0-9.0		6.0-10.0		4.0-6.0	6.0-10.0				
	9.0	11.0-17.0		12.0-15.0		13.0-16.0		4.0-8.0	13.0-16.0				
	3.0-3.5	4.0-6.0		4.0-5.0		4.0-6.0		3.0-4.0	4.0-6.0				
	from now on fed on algae												
May 20	15.0-22.0	17.0-28.0		18.0-23.0		18.0-26.0		7.0-12.0	10.5-20.0				
	5.0-8.0	6.0-10.0		6.0-9.0		6.0-10.0		4.0-5.5	4.5-7.0				
	8.0-11.0	13.0-19.0		12.0-15.0		13.0-17.0		3.5-7.0	6.0-13.0				
	3.0-5.0	3.5-6.0		4.0-5.5		4.0-6.0		2.5-4.0	3.0-4.0				
May 22						18.0-27.0			8.5-19.0	18.0-27.0			
						6.0-10.0			4.5-7.0	6.0-10.0			
						13.0-17.0			3.5-12.0	13.0-17.0			
						4.0-6.0			3.0-4.0	4.0-6.0			

May 21								7.0-11.5	8.0-17.0			
								4.0- 5.5	4.5- 6.5			
								3.0- 6.0	2.5-12.0			
								2.5- 3.0	3.0- 4.0			
May 27	15.0-24.0	18.0-28.5		18.0-24.5		18.0-28.0		7.0-11.5	7.5-17.0	14.0-26.0		
	5.0- 9.0	6.0-10.0		6.0- 9.0		6.0-10.0		4.0- 5.5	4.5- 6.5	6.0-10.0		
	8.0-15.5	13.0-19.0		12.0-16.0		13.0-18.0		3.0- 6.0	2.5-12.0	9.5-16.0		
	3.0- 5.0	3.5- 6.0		3.0- 5.5		4.0- 6.0		2.5- 3.0	3.0- 4.0	3.5- 6.0		
May 31								7.0-11.5	7.5-16.0	12.0-21.0		
								4.0- 5.0	4.5- 6.0	5.0- 8.0		
								3.0- 6.0	3.5-10.0	6.0-14.5		
								2.5- 3.0	3.0- 4.0	3.0- 4.5		
June 5	15.5-25.0	18.0-29.0		18.0-25.0		18.0-30.0			7.0-15.0	9.0-16.0	18.0-30.0	
	6.0-10.0	6.0-10.0		6.0- 9.0		6.0-10.5			3.5- 5.0	4.5- 6.0	6.0-10.5	
	9.0-15.5	13.0-19.0		12.0-16.0		13.0-20.0			3.5-10.0	3.5-10.0	13.0-20.0	
	3.0- 5.5	3.5- 6.0		3.0- 5.5		4.0- 6.0			2.5- 3.5	3.0- 4.0	4.0- 6.0	
June 12										11.0-15.5	15.0-21.0	
										5.0- 6.0	5.5- 7.5	
										5.0-10.0	10.0-14.0	
										3.0- 4.0	3.5- 4.5	
June 17			18.0-29.0		18.0-26.5					9.0-15.0	9.0-17.0	18. -30.0
			6.0-10.0		6.5-10.0					5.0- 6.0	5.0- 6.5	6.0-10.5
			13.0-19.0		12.0-17.0					5.0- 9.0	4.5-11.5	13.0-20.0
			3.5- 6.0		4.0- 6.0					3.0- 4.0	3.0- 4.0	4.0- 6.0
June 21	23.0-25.0	19.0-29.0	13.5-19.0	18.0-28.5	15.0-19.0	18.0-30.0						16.0-22.0
	9.5-10.0	7.0-10.0	6.0- 7.0	6.5-10.0	5.0- 6.0	6.0-10.5						5.5- 7.5
	13.5-15.5	13.0-19.0	7.0-12.5	12.0-18.0	8.5-13.0	13.0-20.0						10.5-11.5
	5.5- 6.0	4.5- 6.0	4.0- 4.5	4.0- 6.0	3.5- 4.0	4.5- 6.0						3.5- 5.0
										one alive		
July 11	23.0-25.0	21.5-31.0		18.0-30.0		18.0-31.0				15.5		11.0
	9.5-10.0	7.5-11.0		7.0-10.5		6.0-11.5				6.5		5.5
	13.5-15.5	14.0-20.0		12.0-20.0		13.0-20.0				9.0		5.5
	6.0	4.5- 7.0		4.0- 6.5		4.5- 8.0				4.0		4.0

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[1] When four measurements are given, the first refers to the entire length, second to length of body, third to length of tail, fourth to breadth of body.

April 21 The thyroid individuals are budding fore legs, the left one first;[1] 16 days old.

April 22 Thyroid ones begin to die.

Only 3 days after the thymus-fed ones were changed to thyroid the influence is noticeable. They have become reduced in size, take on frog shape and bud hind legs.

April 23 Fore legs budding after a treatment of only four days. Thymus-thyroid I do not seem as frail as animals fed on thyroid alone.

April 25 Thyroid-fed animals do not develop their limbs any further, nor do they reduce their tails much further since last observed; progress has ceased.

April 29 The last thyroid and thymus-thyroid I fed animals die.

May 6 A second group of thymus-fed animals is changed to thyroid, thymus-thyroid II.

May 9 Thymus-thyroid II have hind legs appearing and the frog shape is noticeable.

May 10 Thymus-thyroid II have fore legs budding out, the left one first.

May 13 A third group of thymus-fed animals is changed to thyroid, thymus-thyroid III.

May 15 The control animals have not been fed so far. They do not show any sign of differentiation and very little growth. From now they are fed on Elodea canadensis.

May 18 All thymus-thyroid II have both fore legs.

Thymus-thyroid III have hind leg buds.

May 22 All thymus-thyroid III have their hind legs well developed.

Other thymus-fed animals changed to thyroid, thymus-thyroid IV.

May 28 Thymus-thyroid III have fore legs.

Thymus-thyroid IV have hind legs.

June 2 Thymus-thyroid IV have fore legs.

June 5 Thymus-thyroid II begin to die.

June 6 Some muscle-fed tadpoles have hind legs beginning.

June 7 Another group of thymus-fed animals put on a thyroid diet, thymus-thyroid V.

June 12 Thymus-thyroid V have hind legs beginning and the frog shape faintly noticeable.

June 17 Again a group of thymus-fed tadpoles changed to thyroid diet, thymus-thyroid VI.

Part of muscle and spleen fed animals put on a thyroid diet, muscle-thyroid and spleen-thyroid.

June 18 Last thymus-thyroid II dies.

The members of the thymus-thyroid V group have fore leg buds.

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[1] Barfurth states that in 80% of the tadpoles the right fore leg appears first. I have always observed the opposite.

June 19	All thymus-thyroid V die, thyroid had been placed in the water every day.
June 20	Thymus-thyroid VI have hind leg buds.
June 21	Muscle-thyroid have hind leg buds.
June 24	Spleen-thyroid have hind leg buds.
June 25	Thymus-thyroid VI and muscle-thyroid groups have fore leg buds.
June 26	Spleen-fed tadpoles have hind legs.
June 27	Spleen-thyroid group have beginning fore legs.
June 8	Thymus group have hind leg buds.
June 29	Control tadpoles have hind leg buds.
July 3	Last animal of the thymus-thyroid III group dies.
July 6	Last individuals of the muscle-thyroid and spleen-thyroid animals are preserved.
July 10	The remaining thymus-thyroid VI are preserved.
July 14	Last one of the thymus-thyroid IV group dies.
July 20	The experiment is discontinued. The muscle-fed tadpoles are best differentiated.

Since Experiment III was performed along the same lines as Experiment II. it may be well now to give the data of Experiment III and discuss the two groups together.

Experiment III

Rana temporaria, Set III. Figure 3, *a* to *n*. Contained apparently three lots of eggs mixed; they hatched April 7 to 8 and the feedings began April 13, 1912. The original lengths of the tadpoles were from 10 to 11.5 mm.

The experimental data are as follows:

April 13	Food is placed in the dishes for the first time.
April 15	Food is first taken by the animals.
April 18	Thymus-fed specimens become darker in color.
April 20	Some of the muscle- and thymus-fed tadpoles show hind leg buds.
May 6	Part of the thymus-fed animals changed to thyroid diet, thymus-thyroid I.
May 10	All individuals of the thymus-thyroid I group have hind legs and the frog shape is noticeable.
May 13	Thymus-thyroid I group have fore legs budding, the left one first. They lie on their backs and breathe rapidly. A second group of thymus-fed animals is put on thyroid, thymus-thyroid II.
May 15	The control has not yet been fed. It remains far behind the other groups in size and shows no signs of differentiation. From now on the control group is fed on Elodea canadensis.

TABLE 4

Measurements in millimeters during the experiment

DATE	CONTROL	MUSCLE	MUSCLE-THYROID	THYMUS	THYMUS-THYROID I	THYMUS-THYROID II	THYMUS-THYROID III	IV	V
April 13..	10.0-11.5								
Apr. 20	12.0-13.0	14.0-16.0	length	15.0-18.0					
		3.5- 4.0	breadth	3.5- 4.0					
May 6	12.0-14.0[1]	15.0-21.0		15.0-21.0	15.0-21.0				
	3.0- 4.0	5.0- 7.0		5.0- 7.0	5.0- 7.0				
	9.0-10.0	12.0-14.0		12.0-15.0	12.0-15.0				
	2.5- 3.0	3.0- 4.0		3.0- 4.0	3.0- 4.0				
May 13	12.0-15.0	15.0-22.0		15.0-22.0	9.0-14.0				
	3.0- 5.0	5.0- 8.0		5.0- 8.0	4.0- 6.0				
	9.0-10.0	12.0-15.0		12.0-15.0	3.0- 9.0				
	3.0- 4.0	3.0- 5.0		3.0- 5.0	3.0- 4.0				
May 15	from now on fed on algae				9.0-12.0	15.0-22.0			
					3.0- 5.0	5.0- 8.0			
					3.0- 7.5	12.0-15.0			
					2.5- 3.5	3.0- 5.0			
May 18					7.0-12.0	13.0-19.5			
					3.0- 5.0	4.5- 7.0			
					3.0- 7.5	8.5-14.0			
					2.5- 3.5	3.0- 4.0			
May 20	12.0-20.0	16.0-23.0		15.0-22.0	7.0-11.0	9.0-18.0			
	3.0- 8.0	5.0- 8.0		5.0- 8.0	3.0- 5.0	4.0- 6.0			
	9.0-13.0	9.5-15.5		9.0-14.0	3.0- 7.0	4.5-12.0			
	3.0- 4.5	3.0- 5.0		3.0- 5.0	2.5- 3.5	3.0- 4.0			
May 22					7.0-11.0	8.0-16.5	15.0-21.0		
					3.0- 5.0	4.0- 6.0	5.0- 7.5		
					3.0- 7.0	3.5-11.0	9.0-14.0		
					2.5- 3.5	3.0- 4.0	3.0- 4.5		

May 27	14.0-22.0	16.5-25.0		15.0-24.0	7.0-11.0	8.0-16.5	14.0-21.0		
	5.0-8.5	5.0-9.0		5.5-9.5	3.0-5.0	4.0-6.0	5.0-7.5		
	9.0-14.0	11.0-17.0		9.0-16.0	3.0-6.0	3.5-11.0	7.5-13.5		
	3.0-4.5	3.0-5.0		3.0-5.0	2.5-3.5	3.0-4.0	3.0-4.5		
May 31					6.5-10.0	7.0-14.0	11.0-19.5		
					3.0-5.0	3.5-5.5	4.5-6.5		
					2.0-5.0	3.5-9.0	6.0-13.0		
					2.5-3.0	2.5-3.5	3.0-4.0		
June 5	14.0-23.0	16.5-27.0		15.0-25.5	6.5-10.0	8.0-14.0	9.0-18.0	15.0-25.5	
	5.0-9.0	5.0-9.5		5.5-9.5	3.0-5.0	4.0-5.5	4.5-6.0	5.5-9.5	
	9.0-11.5	11.0-19.0		9.0-17.5	2.0-5.0	3.5-9.0	4.0-12.0	9.0-17.5	
	3.0-4.5	3.0-5.0		3.0-5.0	2.5-3.0	2.5-4.0	3.0-4.0	3.0-5.0	
June 12		Short ones die				11.0-14.0	9.0-15.0	12.5-22.0	
						4.5-5.5	4.5-5.5	5.0-8.0	
						3.5-8.5	3.5-10.5	7.5-14.5	
						3.0-3.5	3.0-4.0	3.0-4.5	
June 17	17.0-23.0	18.0-27.0	18.0-27.0	16.0-26.0		11.0-11.0	9.0-15.0	8.0-19.0	16.0-26.0
	7.0-9.0	6.0-10.0	6.0-10.0	6.0-10.0		4.5-5.5	4.5-5.5	3.5-5.5	6.0-10.0
	10.0-11.5	12.5-19.0	12.5-19.0	10.0-17.5		3.5-8.5	3.5-10.5	5.0-11.5	10.0-17.5
	4.0-5.0	4.0-5.5	4.0-5.5	3.5-5.5		3.0-3.5	3.0-4.0	3.0-4.0	3.5-5.5
June 24	21.0-23.0	20.0-28.0	16.0-21.0	17.0-27.0			9.0-15.0		14.0-20.0
	8.5-9.0	6.5-10.0	5.5-7.0	6.0-10.0			4.5-5.5		5.5-7.0
	12.0-14.5	13.5-19.0	11.5-14.0	11.0-17.5			3.5-10.5		8.5-13.0
	4.5-5.5	4.0-5.5	3.5-5.0	3.5-6.0			3.0-4.0		3.5-4.5
July 11	21.0-24.0	21.0-29.0		19.0-31.5			14.0-15.5		8.0-12.0
	8.5-9.0	6.5-10.5		7.0-11.0			5.0-6.5		5.5-6.5
	12.5-15.0	14.5-19.0		12.0-20.5			9.0-9.5		2.5-5.5
	4.5-6.0	4.0-5.5		3.5-6.0			4.0		2.5-3.5

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[1] When four measurements are given, the first refers to the entire length, second to length of body, third to length of tail, fourth to breadth of body.

May 18	The thymus-thyroid I animals are hardly able to swim; when disturbed they move for a few seconds with convulsive jerks, then drop again to the bottom of the dish.
May 19	The thymus-thyroid II group (fed thyroid three times only, May 13, 14 and 17) have hind legs and the frog shape becomes noticeable.
May 22	A third group of thymus-fed animals put on thyroid, thymus-thyroid III.
May 27	Thymus-thyroid II have fore leg buds.
May 28	Thymus-thyroid III (fed four times on thyroid, May 22, 23, 25 and 26) have hind leg buds.
June 2	Thymus-thyroid III have fore leg buds.
June 7	A fourth group of the thymus-fed tadpoles are put on thyroid, thymus-thyroid IV.
June 9	The last specimens of the thymus-thyroid I group are dying.
June 12	The thymus-thyroid IV animals have hind leg buds and the frog-shape is noticeable.
June 17	A fifth group of the thymus fed animals is changed to a thyroid diet, thymus-thyroid V.
	Also some of the muscle-fed ones are put on thyroid.
June 18	The thymus-thyroid IV animals have fore legs, but these specimens are dying.
June 20	Last of the thymus-thyroid II are dying.
June 21	Thymus-thyroid V have hind legs.
June 25	Thymus-thyroid IV have fore legs.
June 26	The muscle-thyroid animals have hind legs.
June 30	The muscle-thyroid animals have fore legs.
July 1	A group of thymus-fed specimens put on a liver diet.
July 4	Some of the thymus-thyroid III group that have been kept on vegetable food since June 5 seem to recover from the thyroid influence.
July 7	The last thymus-thyroid V are dying.
July 14	Muscle-fed tadpoles grow hind legs.
July 20	The experiment is discontinued, and only the muscle-fed animals have extremities.

Experiments II and III were performed for the purpose of determining whether tadpoles of different ages would react to the thyroid diet in similar or different ways. For this purpose a great number of tadpoles were kept on a thymus diet and groups of these were changed to a thyroid diet at various times. Table 5 shows the time of reaction to the thyroid stimulus in the different groups. The number of days in the first columns indicates the respective ages of the animals at the start of the thyroid feed-

TABLE 5

SET IV		SET III	
14 days	hind 3 days		
	fore 4 days		
30 days	hind 4 days	28 days	hind 4 days
	fore 6 days		fore 7 days
37 days	hind 6 days	35 days	hind 6 days
	fore 15 days		fore 14 days
46 days	hind 6 days	44 days	hind 6 days
	fore 11 days		fore 10 days
62 days	hind 5 days	60 days	hind 5 days
	fore 11 days		fore 11 days
72 days	hind 3 days	70 days	hind 4 days
	fore 8 days		fore 8 days

ings. The figures in the other columns give the number of days required by each set in developing hind and fore legs.

The similarity of the results in the two series is striking. There is a gradual decrease in the rapidity of action of the thyroid influence up to the age of about 5 weeks followed by a steady increase after this time. Whether or not this is a general rule must be decided by further experiments. It is also difficult to give a satisfactory explanation for this phenomenon, yet the following suggestion may be advanced. The young tadpoles were very quickly affected by the thyroid feedings because the previous thymus diet had not acted long enough to delay or counterbalance the thyroid stimulus. Older animals had enough thymus *agens* accumulated to retard the thyroid action. Still older tadpoles were more nearly approaching the normal time of metamorphosis and may thus have been ripe to respond to an accelerating stimulus. The last argument, however, seems especially weak, since it introduces a new factor in the action of thymus diet; namely, that if it is prolonged, it loses the retarding influence and thus is less able to counteract the thyroid. This factor has not yet been demonstrated; thus no conclusions as to the rapidity of the thyroid influence on different age tadpoles can be based upon it.

It has been shown, however, in the experiments of 1911 that thymus will affect older tadpoles less than younger ones. Another point may also be mentioned without attempting to explain the situation. Table 6 shows that old thymus-fed tadpoles, when put on a thyroid diet, respond more quickly to the thyroid stimulus than do tadpoles of the same age which have been fed on other substances. One should have expected just the contrary, judging especially from Experiment VI in 1911, in which liver-thyroid-fed tadpoles developed their fore legs 3 days earlier than thymus-thyroid-fed ones.

TABLE 6

	SET IV: 72 DAYS OLD			SET III: 70 DAYS OLD	
	Thymus-thyroid	Muscle-thyroid	Spleen-thyroid	Thymus-thyroid	Muscle-thyroid
Hind legs in	3 days	4 days	7 days	4 days	9 days
Fore legs in	8 days	8 days	10 days	8 days	13 days

In Experiments II and III those tadpoles (fig. 3 *c* to *h*, 3 *k*, 3 *m*, 3 *n*; fig. 4 *e* to *i*, 4 *l*, 4 *n* to *p*) that were transferred from thymus to thyroid diets were fed on the latter gland only three or four times. When they had developed their fore legs water-plants were placed in the dishes. It had been noticed that thyroid-fed tadpoles would die very soon after putting out fore legs. In these experiments those that were placed in dishes containing plants, although they were never seen to feed on the plants, could be kept alive for some time, 20 to 53 days, while those specimens remaining in water in which thyroid was placed longer than was absolutely necessary for developing the extremities died within 10 to 12 days after the beginning of the thyroid diet. Those living longer did not develop any further than those dying early, but remained stationary except for a further reduction in size, especially of the tail.

Table 7 gives the length of time that the different thymus-thyroid-fed sets were kept alive in the two experiments. Tadpoles in Experiment III were about 2 days younger than those in Experiment IV, when the feeding began.

TABLE 7

SET IV		SET III	
Age at the time thyroid was given	Days living after thyroid treatment	Age at the time thyroid was given	Days living after thyroid treatment
14 days	10 days (no plants)		
30 days	43 days	28 days	33 days
37 days	51 days	35 days	38 days
46 days	53 days	44 days	50 days
62 days	12 days (no plants)	60 days	11 days (no plants)
72 days	23 days	70 days	20 days

A glance at table 5 will show that the tadpoles of 37 and 46 (or 35 and 44) days showed the slowest reaction to the thyroid treatment. The possible reasons for this behavior were discussed above.

Experiment IV

Rana temporaria. Set II. Figure 2, *a* to *n*. 960 tadpoles hatched on April 6 to 7, 1912, and fed on muscle until June 3. The experiment started on June 5.

This experiment confirmed the results of the two previous ones. Tadpoles (fig. 2, *a*) which had lived for 50 days on an ordinary meat diet were later affected by the thyroid stimulus as greatly as animals fed only on a thyroid diet. The main object of this experiment, however, was to ascertain how long the thyroid *agens* had to be applied before results were noticeable, and furthermore, whether or not it would be possible to counteract the thyroid influence after one or more days feeding. A great number of tadpoles were reared on muscle and at the age of 50 days were divided into groups of 80 individuals each. The different groups were then fed on thyroid from 1 to 5 days and after this treatment five of the groups were put on a vegetable diet (Elodea canadensis) (fig. 2, *f* to *i*) and 5 others on muscle diet (fig. 2, *c* to *e*).

One group (fig. 2, *b*) was allowed to starve to test whether hunger following a long period of feeding (over 6 weeks) would bring about a quicker differentiation.

Still another group (fig. 2, *l*) was kept in water in which small pieces of thyroid gland were placed, but so arranged that the

TABLE 8

Measurements in millimeters and diary notes

DATE	CONTROL FED	PLANTS AFTER A THYROID DIET OF					MUSCLE AFTER A THYROID DIET OF					THYROID EMULSION
		1 day	2 days	3 days	4 days	5 days	1 day	2 days	3 days	4 days	5 days	
June 5.					14.0–22.0 mm[1] 4.5–9.0 mm 9.0–15.0 mm 3.5–5.5 mm							
					After a few days' feeding the shorter individuals die							
June 10..					Hind			legs				
June 11..			Hind legs									
	14.5–22.0 4.5–9.0 9.0–15.0 3.0–5.5	16.0–22.0 5.0–8.5 10.5–14.0 3.0–5.0	9.5–19.0 4.5–6.5 4.0–12.5 3.0–4.0	11.0–19.0 4.5–6.5 6.0–13.5 3.0–4.0	15.0–20.0 5.0–7.0 8.0–13.5 3.0–4.5	14.0–17.0 5.0–6.5 7.5–11.5 3.0–4.5	17.0–22.0 6.0–8.5 6.5–15.0 4.0–5.0	15.0 6.5 8.5 4.0	17.0–19.0 6.0–7.0 11.0–12.5 4.0–4.5	11.0–18.0 5.5–7.0 4.5–11.5 4.0–4.5	15.5–19.0 5.5–7.0 9.0–13.0 3.0–4.0	14.0–22.0 4.5–9.0 9.0–14.0 3.0–5.0
June 12		Frog-shape faintly noticeable						Only 1 living	About 80% have died			
June 13					Frog-shape noticeable					Fore	legs	Hind legs frog shape
June 14			Fore legs					Last one dead				
June 15..					Fore legs							

June 18	16.0-21.0	16.0-22.0	8.0-16.0	10.5-18.0	9.5-19.0	10.0-17.0	17.5-28.0		17.0-18.0	9.5-12.5	9.0-18.0	12.0-17.0
	5.5- 9.5	5.0- 8.5	4.5- 6.5	4.5- 6.5	4.0- 7.0	4.5- 6.5	7.0- 9.5		6.0- 6.5	5.5- 6.0	5.0- 6.0	4.5- 6.0
	11.0-16.0	10.5-11.0	1.5-10.0	6.0-12.0	5.0-13.0	5.0-10.5	10.5-18.5		11.0-11.5	3.5- 7.0	2.5-12.0	7.5-11.0
	3.0- 5.5	3.0- 5.0	3.0- 4.0	2.5- 4.0	3.0- 4.5	3.0- 4.0	4.0- 6.0		4.0	3.0- 3.5	3.0- 3.5	3.0- 4.0
June 19		Hind legs	A great number die				About 10% living		About 10% living	4% living		11.0-13.0 4.5- 5.5 6.5- 8.0 3.0- 3.5
												All dead
June 25	18.0-27.0	17.5-25.0	15.0-18.0	9.0-16.0	11.0-19.0	10.0-14.0	18.0-31.0		18.0-19.5	9.0-11.5	10.0-17.0	
	6.5-10.0	6.0- 9.5	5.0- 6.0	3.5- 5.5	1.5- 7.0	1.0- 6.0	7.0-11.0		6.0- 7.5	4.0- 6.0	4.5- 5.5	
	11.5-17.0	11.0-16.0	10.0-12.0	5.5-10.5	6.5-12.0	5.0- 9.0	11.0-20.0		12.0-12.5	3.0- 7.0	5.5-11.5	
	3.5- 6.0	3.5- 6.0	3.0- 4.0	2.5- 3.5	3.0- 4.5	3.0- 3.5	4.0- 6.5		4.5	3.0- 3.5	3.0- 3.5	
July 2.						Last one dead						
July 4...									1 living	Last ones dead		
July 5...	Hind legs											
July 8...			Last ones dead									
July 11	18.0-30.0	19.0-27.0			16.0-21.0		19.0-33.0		24.0			
	6.5-11.5	7.0-10.0			6.5- 7.5		8.0-10.5		8.5			
	11.5-18.5	12.0-17.0			9.5-14.0		11.0-22.5		15.5			
	3.5- 6.5	4.0- 6.0			3.0- 4.5		5.0- 6.5		5.0			
July 15		5 living			1 living		4 living; fore legs		1 living			

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[1] When four measurements are given, the first refers to the entire length, second to length of body, third to length of tail, fourth to breadth of body.

animals could not feed on it. The gland was placed in a small glass vial closed with gauze and allowed to stand upright, the open end being beneath the surface of the water. Thus no thyroid particles could get out into the water, merely an extract or emulsion of thyroid constituents which diffused from the vial into the dish. Animals kept in these dishes developed their hind legs 8 days after the beginning of the experiment and the frog shape became noticeable about the same time. Six days later they all died without having grown fore legs, but their size on that day, June 19, shows that they were completely under the thyroid influence. Therefore the product of the thyroid which caused the rapid differentiation in all the previous experiments must be soluble in water.

The control animals which were kept starving after the 50 days feeding did not grow hind legs until July 5, considerably later than the thyroid treated ones. Thus the results after thyroid application are different from those Barfurth reports in his "Der Hunger als förderndes Prinzip in der Natur," and cannot be attributed to starvation of the tadpoles. Among the ten main groups of the experiment the thyroid influence manifested itself in different ways. As a general result it may be stated that a thyroid application of only 24 hours sufficed to show decided effects (fig. 2 *c*, 2 *f*), and that a treatment of more than 24 hours (2 to 5 days) gave the typical results described in all previous experiments.

In the five groups that received vegetable food following the thyroid dose and in the five groups that were fed again on muscle, a great number of individuals, 50 to 60 per cent, mainly the shorter ones, died within 2 or 3 days after the first feeding. This accords with observations in other cases where thyroid was given after previous feeding, but is in striking contrast with the happenings in experiments where thyroid food was applied from the start. In the latter experiments very few animals were lost during the first days. It remains to be answered why the application of thyroid should affect tadpoles that had not been previously fed on other diets less harmfully than those previously fed on various tissues.

The tadpoles which again received muscle after the thyroid treatment reacted more strongly to the stimulus than those put on a vegetable diet. They all grew hind legs within from 1 to 5 days, the frog shape became noticeable very soon, and they died in great numbers, so that 7 days after the beginning of the experiment only 20 per cent were alive. The animals treated from 3 to 5 days grew fore legs on the 8th day, those treated 2 days were all dead on the 9th day; this group was affected most strongly, which may be a mere coincidence. The groups fed only one day grew fore legs on the 42d day, at this time only four of this group were alive, only one of the 3 day group, and none of the 4 and 5 day groups, the last ones having died on the 29th day of the experiment. That is, out of 400 individuals only 4 in the 1 day group and 1 in the 3 day group were able to survive the thyroid shock. The table of growth on page 450 shows that these surviving individuals actually begin to grow again. These 4 animals of the 1 day group seem to have absorbed a very small quantity of the thyroid *agens*. They stop their precocious differentiation very soon and do not grow fore legs until July 17, which is 34 days later than the 3 and 5 day groups.

The second set of tadpoles, those put on a vegetable diet after a thyroid treatment, reacted in a similar fashion to that just described, except perhaps as stated above, they were somewhat less affected. This might suggest that a meat free diet may help to counteract thyroid influences. The 3 and 5 day groups grow hind legs on the 5th day of the experiment, the 2 day group on the 6th day, and the 1 day group not until the 14th day. Fore legs appear in the 3, 4 and 5 day groups on the 10th day, in the 2 day group on the 9th day, in the 1 day group none had appeared on the 46th day, when the experiment was discontinued. While many animals died during the first few days, some were able to keep alive. On the 27th day the last ones of the 5 day group died, and on the 33d day all of the 2 and 3 day groups were dead. There were five of the 1 day group and one of the 4 day group alive on the 42d day. These, as the table of growth of page 450 shows, start again to grow.

TABLE 9

Measurements in millimeters and diary notes

DATE	CONTROL UNFED	AQUARIUM	BLOOD	PANCREAS	PANCREAS EMULSION	SPLEEN	SPLEEN EMULSION	LIVER	LIVER EMULSION	TESTICLE	TESTICLE EMULSION	OVARY	OVARY EMULSION	ADRENAL	ADRENAL EMULSION	THYMUS	THYMUS EMULSION	THYROID	THYROID EMULSION
May 8..										13- 18 mm.[1] 4- 6 mm. 8 12 mm. 3- 4 mm.									
May 12										Many animals die in the emulsions								Last thyroid feeding	
May 13...																		Hind legs noticeable	
May 15	13.0-17.0	13.0-21.0	13.0-18.0	13-17		13-19		13.0-19.0	13.0-15.0	13.0-18.0		13.0-18.0		13.0-18.5	13.0-15.0	13.0-19.0	13.0-18.0	10.0-15.0	12.0-16.0
	4.0-6.0	5.0-8.0	4.0-6.0	4-6		4-7		4.0-7.0	4.0-6.0	3.0-4.5		4.0-6.0		4.0-6.5	4.0-6.0	4.0-7.0	4.0-6.0	4.0-5.0	4.0-6.0
	8.0-12.0	8.0-11.0	8.0-12.0	8-12		8-12		8.0-12.0	8.0-10.0	8.0-12.0		8.0-12.0		8.0-12.0	8.0-11.0	8.0-12.0	8.0-12.0	5.0-10.0	6.0-10.0
	3.0-4.0	3.0-5.0	3.0-4.0	3-4		3-4		3.0-4.0	3.0-4.0	3.0-4.0		3.0-4.0		3.0-4.5	3.0-4.0	3.0-4.0	3.0-4.0	3.0-4.0	3.0-4.0
				Do not appear to take food, many dying															
May 18.																		7.0-14.0	10.5-15.0
																		3.5-4.5	3.5-5.5
																		4.5-10.0	7.0-10.0
																		2.5-3.0	2.5-3.5
																		Left fore leg	
May 20.																		7.0-14.0	7.5-14.0
																		3.5-4.5	3.5-5.5
																		4.5-10.0	4.0-9.0
																		2.5-3.0	2.5-3.5
																		Transferred into aerated water	
May 22	13.0-17.0	17.0-27.0	13.0-21.0	13.0-21.0		14.0-21.0	14.5-21.0	13.0-23.0	14.0-20.0	13.0-22.0	14.0-23.0	13.0-22.0	14.0-22.0	13.0-22.0	13.5-21.5	13.0-23.0	13.0-21.0	7.0-12.0	7.5-14.0
	4.0-6.0	6.5-11.0	4.5-7.0	4.5-8.0		5.0-8.0	5.0-8.5	4.5-9.0	4.5-7.0	4.0-8.0	5.0-9.0	5.0-9.0	5.0-8.5	4.5-9.0	4.5-8.0	5.0-9.0	4.0-8.0	3.5-4.5	3.5-5.5
	8.0-12.0	11.0-17.0	8.0-14.0	10.0-11.5		9.0-13.5	8.5-16.0	9.0-14.0	9.5-14.0	8.0-14.0	8.5-14.0	9.0-14.0	9.0-14.0	8.5-14.0	8.5-13.5	8.0-14.0	8.0-14.0	3.5-9.0	4.0-9.0
	3.0-4.0	4.0-6.0	3.0-4.0	3.0-5.0		3.0-5.0	3.0-5.0	3.0-5.5	3.0-4.0	3.0-5.0	3.0-5.0	3.0-5.0	3.0-5.0	3.0-5.0	3.0-5.0	3.0-5.5	3.0-5.0	2.5-3.0	2.5-3.5
				Only 3 living															
May 24...									Many of the short individuals begin to die										
May 26																		Fore legs	
May 31.	14.0-18.0	17.0-29.0	13.0-24.0	16.0-21.0		14.0-23.0	15.0-25.0	13.0-25.0	19.0-21.0	13.0-23.0	14.0-24.0	16.0-23.0	14.0-22.0	13.0-23.0	15.0-25.0	13.0-21.5	13.0-22.0	6.0-12.0	6.0-13.0
	5.0-6.0	6.5-11.0	5.0-8.0	6.0-8.0		5.0-9.0	5.0-9.0	5.0-10.0	7.0-8.0	4.0-9.0	5.0-10.0	5.0-9.0	5.0-8.5	4.5-10.0	6.0-9.0	5.0-9.0	4.0-8.0	2.5-5.0	3.0-5.0
	8.0-13.0	11.0-18.0	8.5-16.0	10.0-11.5		9.0-14.5	9.0-16.0	9.0-15.0	12.5-14.0	8.0-15.0	8.5-16.0	10.0-15.0	9.0-14.0	8.5-14.0	9.5-16.5	8.0-15.5	8.0-15.0	2.0-8.0	2.5-9.0
	3.0-4.0	4.0-6.0	3.0-5.0	4.0-5.0		3.0-5.0	3.0-5.5	3.0-6.0	4.0-4.5	3.0-5.5	3.0-5.0	3.0-6.0	3.0-5.5	3.0-6.0	3.0-5.0	3.0-5.5	3.0-5.0	2.0-3.0	2.5-3.0

June 1																	Last ones dead
June 12	Died June 7th.	15.5-29.0 5.5-10.5 10.0-19.0 4.0-6.0	14.5-28.0 5.0-10.0 9.0-18.0 3.0-5.0	21.0 7.0 14.0 4.5		14.0-26.0 5.0-9.5 9.0-17.0 3.0-5.5	15.5-27.5 5.5-9.5 10.0-18.0 3.0-5.5	16.0-27.0 5.0-10.5 11.0-17.0 3.5-6.5	22.0-26.5 8.0-9.5 14.0-17.0 4.5-5.5	14.0-26.5 5.0-10.0 8.0-16.5 3.0-5.5	15.0-26.5 5.5-10.0 9.5-16.5 3.5-6.0	17.0-26.5 5.5-10.0 10.0-16.5 3.0-6.0	14.5-27.5 5.5-9.5 9.0-17.5 3.5-5.5	14.0-24.0 5.0-9.0 9.0-15.5 3.5-6.5	15.5-29.0 5.5-10.0 10.0-19.0 3.5-6.5	14.0-25.5 5.5-9.5 9.0-16.5 3.5-5.5	14.0-26.0 5.0-9.5 9.0-16.5 3.5-6.5	
June 19.		Hind legs																
June 20...			Hind legs					Hind legs				Hind legs						
June 24		15.5-30.0 5.5-11.0 10.0-19.0 4.0-6.0	17.0-31.5 6.0-11.0 11.0-20.5 4.0-6.5	21.5 7.0 14.5 4.5		16.0-27.5 6.0-10.0 10.0-18.0 3.5-6.0	17.5-30.0 6.0-10.0 11.5-20.0 4.5-6.0	17.0-30.0 6.0-11.0 11.0-20.0 4.5-6.5	25.0-27.5 8.0-9.5 17.0-18.0 5.0-5.5	16.5-32.0 6.0-11.0 10.5-21.0 4.0-6.5	15.5-28.0 5.5-10.5 10.0-17.5 3.5-6.0	17.0-31.0 6.0-10.5 11.0-20.5 4.0-7.0	16.5-30.5 5.5-10.0 11.5-20.5 3.5-6.5	18.0-28.0 7.0-12.0 11.0-16.0 5.0-8.0	19.0-32.0 7.0-10.5 12.0-21.5 4.0-6.5	15.0-27.0 5.5-9.5 9.5-17.5 3.5-5.5	18.0-30.0 7.0-10.0 11.0-20.0 4.0-6.5	
June 26...										Hind legs				Hind legs				
June 28...																Hind leg buds		
July 5....			Fore legs					Fore legs										
July 8..			First metamorphosis	Dead														
July 11		18.0-30.0 7.0-11.0 11.0-19.0 4.5-6.0	17.0-31.5 6.0-11.0 11.0-20.5 4.0-6.5			16.0-31.5 6.0-11.0 10.0-20.5 3.5-6.5		17.0-30.0 6.0-11.0 11.0-20.0 4.5-6.5		16.5-33.5 6.0-11.5 10.5-22.0 4.0-7.5		20.0-33.5 7.0-11.0 13.0-22.5 4.0-7.0		18.0-34.0 7.0-12.0 11.0-22.0 5.0-8.0		19.0-31.0 7.0-11.0 12.0-20.0 4.0-6.5		
July 12.								First metamorphosis										
July 14...												Fore legs						
July 18.						Hind leg buds												
July 19...										Fore legs								
July 21.												First metamorphosis						
July 26.										First metamorphosis								

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[1] When four measurements are given, the first refers to the entire length, second to length of body, third to length of tail, fourth to breadth of body

Experiment V

Rana temporaria, Set VIII to XI, voluntarily mixed. These hatched April 10 to 13 and the experiment started May 8, 1912.

In addition to the organs used in the previous experiments, pancreas and testicle were given as food in this experiment. Each organ was given in two forms; with one group of tadpoles a small piece of the tissue was placed in the water; to a second group the food was given in a crushed form, a piece of the tissue being squeezed between the fingers, until it was broken into minute particles. This was done for two reasons. First to allow juices that might form an active part of the glands to ooze out into the water as quickly as possible, second to give the weaker individuals a chance to find food particles. In almost every experiment it was seen that a number of the smallest individuals did not grow and after a time began to die. It seems, however, that this is not due to an inability to obtain food, but because they were less fit to live and were simply weeded out in the early stages of development.

The tadpoles fed on the crushed glands developed in essentially the same manner as the others, thus after several weeks both sets were placed in one dish and fed alike.

One set of animals was fed on clotted blood. They did best of all. A control set was fed on Elodea canadensis and another was starved.

All of the tadpoles were about 4 weeks old at the beginning of the experiment and had not been fed. When starved the animals eat the dead ones, if these are not removed, but their rate of growth can by no means compare with that of tadpoles receiving plenty of food.

The thyroid-fed tadpoles showed the same rapid development as observed in previous experiments. They could not be kept alive longer than 23 days, although after 10 days they were put in water through which a constant current of air was passed. The groups fed on other tissues also showed the usual rate of growth. These individuals fed on spleen, liver and thymus ran rather parallel courses and progressed somewhat faster during the early days of the experiment than the tadpoles fed on testicle, ovary and adrenal. The latter three groups also ran similar courses. The set fed on plants showed a rather rapid growth, about equal to that of the spleen, liver and thymus groups. The tadpoles in these groups reached on the average a length of 30 to 31 mm at the time of metamorphosis, while the specimens in the slower growing groups reached a length of 33 to 34 mm before they metamorphosed. This fact might lead one to conclude that the materials fed to the faster groups, vegetable, blood, spleen, liver and thymus, contain an *agens* which causes a more rapid differentiation than the foods given to the second groups, testicle, ovary, adrenal. This, however, is not the case, since in the first group the spleen- and thymus-fed tadpoles exhibited no signs of an approaching metamorphosis at a time when the tadpoles of the second group had begun to leave the water.

Attention should be called to the fact that in this experiment as in the previous ones of 1912 the spleen- and thymus-fed groups (spleen had not been fed in 1911) showed almost parallel courses of development, neither reaching the stage of metamorphosis when the other groups had. Whether or not this indicates an influence of the spleen, or perhaps of all lymphatic organs, similar to that of the thymus in retarding differentiation I am at present unable to decide.

The tadpoles intended for a pancreas diet did not feed freely and most of them died early.

The starved animals did not differentiate beyond their original stage and began to die about 4 weeks after the beginning of the experiment.

The pigmentation exhibited by the animals was very much the same as in previous experiments. The thymus-fed individuals, however, did not become as dark as usual. Those fed on blood and plants became very dark.

Experiment VI

Rana temporaria, Set V to VII. Figure 5, *a* to *e*. Probably three sets mixed. They hatched April 6 to 8 and were not fed until May 24, when the experiment began.

During the early part of the season hypophysis was not available, so that no experiments with it could be carried out simultaneously with the other. Therefore this late experiment of hypophysis feeding is not of great significance. It is reported here to show the rates of growth of tadpoles fed on glandular and others on the nervous parts of the hypophysis.

TABLE 10

Measurements in millimeters, and diary

DATE	CONTROL VEGETABLE	HYPOPHYSIS GLANDULAR	HYPOPHYSIS NERVOUS
May 24........	19.0–21.0[1]	17.0–26.0	17.0–26.0
	7.0– 8.0	6.0– 9.0	6.0– 9.0
	11.0–13.0	10.5–16.0	10.5–17.0
	3.5– 4.5	4.0– 6.0	4.0– 6.0
May 31........	19.0–22.0	17.0–26.5	17.0–28.0
	7.0– 8.5	6.5–10.0	6.0–10.0
	11.0–13.5	10.5–18.0	11.0–18.5
	4.0– 5.0	4.0– 6.0	4.0– 6.0
June 7.........	19.0–22.5	17.0–27.5	17.0–29.0
	7.0– 8.5	6.5–10.0	6.0–10.0
	11.0–14.0	11.0–18.5	11.0–19.0
	4.0– 5.0	4.0– 6.0	4.0– 6.0
June 24........	23.0–25.0	17.5–30.5	17.0–30.5
	8.0– 9.5	6.5–11.5	6.0–11.0
	15.0–15.5	11.0–19.0	11.0–19.5
	5.0– 6.0	4.5– 7.0	4.0– 7.0
June 26........		Hind legs Extremely voluminous bodies.	
June 30........			One individual grows faster than the others and develops hind legs
			32.0
			11.0
			21.0
			7.0
July 11........	25.0–27.0	19.5–30.5	20.0–32.0, 34
	9.0–10.0	7.0–11.5	7.5–11.0, 11
	16.0–17.0	12.5–19.0	12.5–21.5, 23
	5.0– 6.0	4.5– 7.0	4.5– 6.5, 7
		Short, but very strong extremities. Begin to die	Only one, the biggest, has hind legs, but still drawn close to the body

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[1] When four measurements are given, the first refers to the entire length, second to length of body, third to length of tail, fourth to breadth of body.

The difference between the rates of growth of the two sets is not marked, there may be a little faster growth in the set fed on neural hypophysis. The tadpoles fed on glandular hypophysis (fig. 5 *b*, 5 *d*) show decidedly better differentiation and gradually become less pigmented than those fed on neural hypophysis (fig. 5 *e*).

Experiment VII

Bufo vulgaris, Set I. Figure 8, *a* to *d*. Hatched April 10 to 20, 1912, and the feeding began May 6.

The experimental data are as follows:

May 5 The feeding began.
May 11 All the thyroid-fed specimens have hind legs and the frog shape is noticeable. They were fed only four times.
May 18 Thyroid-fed animals have developed fore legs.
May 22 Algae were placed in with the thyroid-fed tadpoles.
June 18 Last thyroid-fed ones die.
June 20 Thymus-fed ones have hind leg buds.
June 24 Control animals have hind leg buds.
July 11 The muscle-fed tadpoles have the best differentiated hind legs.
July 14 Muscle-fed animals grow fore legs.
July 20 The first metamorphosis in muscle-fed group. The control still have very small hind leg buds. Thymus-fed animals have their hind legs still drawn close to the body.

This experiment introduced a new species into the study, Bufo vulgaris, yet the results, as one might have expected, are essentially the same. The thyroid-fed group (fig. 8, *d*) showed their usual precocious differentiation, their decrease in size is not as marked as in the Rana species. The thymus-fed group (fig. 8, *c*) did not grow much faster than the muscle-fed ones (fig. 8, *b*) and towards the end of the experiment they actually fell behind in size, yet they showed little, if any, differentiation at the time of the first metamorphosis of the muscle-fed tadpoles. A considerable number of the thymus group died when they reached a length of about 21 mm (status thymicus, thymus death).

TABLE 11

Measurements in millimeters

DATE	CONTROL VEGETABLE	MUSCLE	THYMUS	THYROID
May 6.......	13–16 mm.[1]			
	5– 7 mm.			
	7– 9 mm.			
	3– 5 mm.			
May 13........	14.0–16.0	15.0–17.0	14.0–17.0	13.0–15.5
	5.0– 7.0	6.0– 8.0	6.0– 8.0	5.0– 7.0
	7.0– 9.0	8.0–10.0	8.0–10.0	7.0– 9.0
	3.0– 5.0	4.0– 5.0	4.0– 5.0	3.0– 4.0
				Legs 0.8
May 15........				11.5–15.5
				5.0– 6.5
				5.0– 9.0
				3.0– 4.0
May 18.......				6.5–15.5
				4.0– 6.5
				2.5– 9.0
				3.0– 4.0
May 20........				11.0–15.5
				4.5– 6.5
				6.5– 9.0
				3.0– 4.0
May 22........	14.0–19.0	16.0–19.0	14.0–20.0	11.0–15.5
	5.5– 8.0	7.0– 9.0	6.0– 9.5	4.5– 6.5
	7.5–11.0	9.0–11.0	8.0–11.5	5.0– 9.0
	3.5– 5.0	4.0– 5.0	4.0– 6.0	3.0– 4.0
May 30........	14.0–20.0	16.0–20.0	14.0–20.0	10.0–15.0
	5.5– 8.0	7.0– 9.0	6.5– 9.5	4.5– 6.5
	7.5–12.0	9.0–11.5	8.5–11.5	5.0– 9.0
	4.0– 5.0	4.0– 5.0	4.0– 6.0	3.0– 4.0
June 6.........	14.0–20.0	16.0–20.0	14.0–21.0	10.0–14.5
	5.5– 8.0	7.0– 9.0	7.0– 9.5	4.5– 6.0
	7.5–12.0	9.0–11.5	8.5–11.5	5.0– 8.5
	4.0– 5.0	4.0– 5.0	4.0– 6.0	3.0– 4.0
June 18........	16.0–20.0	16.0–20.0	14.0–21.5	10.0–13.0
	6.5– 8.5	7.0– 9.0	7.0– 9.0	4.5– 6.0
	8.0–12.0	9.0–11.5	8.5–12.0	4.0– 7.0
	4.0– 5.5	4.0– 5.0	4.0– 6.0	3.0– 4.0
June 25........	17.0–20.5	16.0–21.0	17.5–22.0	
	6.5– 8.5	7.0– 9.0	7.0– 9.5	
	9.0–12.0	10.0–12.0	10.5–12.5	
	4.0– 5.5	4.0– 5.5	4.0– 6.5	
July 11........	18.0–21.0	18.0–24.5	19.0–22.5	
	6.5– 9.0	7.5–10.5	8.0– 9.5	
	10.0–12.0	10.5–13.0	11.0–13.0	
	4.5– 6.0	4.0– 6.0	4.5– 6.5	

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[1] When four measurements are given the first refers to the entire length, second to length of body, third to length of tail, fourth to breadth of body.

The next three experiments to be described were performed for the purpose of studying the influence of a mixed diet on growth and development. One kind of food was given one day and another the next. Previously the animals had been fed for a considerable length of time on one kind of tissue and were then transferred to another.

Experiments VIII and IX consider a thyroid-thymus diet only. The tadpoles of Experiment VIII had been fed on muscle before the thyroid-thymus treatment began, while those of Experiment IX had starved. It may be well at present to record the notes

TABLE 12

Measurements in millimeters and diary notes

June 5	21.0–28.0 8.5–10.5 13.0–19.0 4.5–6.0
June 10	Hind legs appear
June 12	20.0–24.0 7.0–8.0 12.0–16.0 4.5–5.5 The frog shape is noticeable
June 18	14.0–24.0 5.5–8.0 9.0–16.0 3.5–5.0 Very active; legs are very thin (fig. 2 *m*), but much better developed than in thyroid (Experiment IV, fig. 4 *p*). Length of the hind legs 2.5 to 3.5 mm; the abdomen is bloated.
June 19	About 50% die
June 25	14.0–22.0 5.5–7.5 8.5–14.5 3.5–5.0 Fore legs appear 10 days later than in the all-thyroid group (Experiment IV).
July 7	14.0–20.0 5.5–7.5 8.5–12.5 3.5–4.5 Last ones preserved

from these experiments and later discuss them in connection with Experiment X, in which several kinds of foods in various combinations were applied.

TABLE 13

Measurements in millimeters and diary notes

	CONTROL (VEGETABLE)	THYMUS-THYROID
June 5....	14.0–22.0 5.0– 8.0 9.0–14.0 3.0– 4.0	
June 12.........	15.0–25.0 5.5– 8.5 10.0–17.0 3.5– 4.5	14.0–21.0 5.0– 7.0 9.0–14.0 3.0– 4.0
June 14 .		Hind legs appear Many die
June 18.........	15.5–26.0 5.5– 9.0 10.5–17.0 3.5– 5.0	14.0–20.0 5.0– 7.0 9.0–13.0 3.0– 4.0 Fore legs appear Very active The abdomen is bloated
June 24.........	16.0–26.0 5.5– 9.0 10.5–17.5 3.5– 5.5	14.0–18.5 5.0– 6.0 9.0–11.5 3.0– 4.0
July 8............	Hind leg buds	
July 13...........		Last one dies

Experiment VIII

Rana temporaria, Set II. Figure 2, *k*, 2, *m*. Hatched April 6 to 7, 1912 (Experiment IV), and fed on muscle until June 3. The largest individuals of Set II were selected for this experiment. The feeding of thyroid-thymus began on June 5.

Experiment IX

Rana temporaria, Sets V to VII. Figure 6, *a* to *d*. Probably three sets mixed. Hatched April 6 to 8, 1912, and not fed until June 5, when the experiment was started.

Experiment X

Bufo vulgaris, Set III. Figure 9, *a* to *r*. Brought into the laboratory May 21, 1912, size 18 to 23 mm, age unknown. Experiment started June 5. Thirteen groups of tadpoles, 150 individuals in each, were given food as indicated below; one group was starved.

A part of this experiment corresponds to Experiment IV in which several groups of tadpoles of Rana temporaria were fed on thyroid for from 1 to 5 days respectively and afterwards put on a vegetable diet. The Bufo tadpoles reacted very quickly to the thyroid stimulus, but when the thyroid feeding was stopped, they seemed to overcome the thyroid influence more readily than any other tadpoles. The groups fed on thyroid from 3 to 5 days (fig. 9, *b* to *d*) developed hind legs 5 days after the first feeding, showing as in other experiments, that a 3 day feeding of thyroid gland suffices to give the typical results. The 2 day group budded the hind extremities on the 7th day and the 1 day group on the 11th day. These intervals approach close to those observed in Experiment IV, which were 5, 6 and 14 days. The anterior extremities appear in the 5 day group 14 days after the first feeding (in Experiment IV on the 10th day) and this group begins to undergo metamorphosis on the 18th day. In the other, 1 to 4 day groups, the after-treatment with vegetable food seems to check the hastened differentiation following the intake of thyroid tissue. They finally do not go much faster than tadpoles which had not received thyroid. It might thus appear as if the Bufo tadpoles had a stronger resistancy against the thyroid stimulus than the Rana larvae. This point is not entirely clear, however, since the former had not received any food before the thyroid feedings began, while the latter had previously lived for 50 days on muscle. As has been stated above, any meat diet before or after the thyroid treatment is apt to render the animals more susceptible to the thyroid stimulus than does starvation.

The thyroid treated Bufo tadpoles do not reduce their size so much as the Rana tadpoles, as before mentioned under Experiment VII.

TABLE 14

Measurements in millimeters and diary

DATE	CONTROL UNFED	TESTICLE	PLANTS AFTER THYROID DIET OF					THYROID				THYMUS		HYPOPHYSIS VEGETABLE
			1 day	2 days	3 days	4 days	5 days	Thymus	Muscle	Hypophysis	Vegetable	Hypophysis	Vegetable	
June 5							18-23 mm.[1] 7- 9 mm. 10-14 mm. 4- 6 mm.							
June 10							Hind legs							
June 12	18.0-23.0 7.0- 9.0 10.0-14.0 4.0- 6.0	18.0-23.0 7.0- 9.0 10.0-14.0 4.0- 6.0	18.0-21.0 7.0- 9.0 10.0-12.0 4.0- 5.5	18.0-22.0 7.0- 9.0 10.0-13.0 4.0- 5.5	18.0-22.5 7.0- 9.0 10.0-14.0 4.0- 5.5	18.0-22.5 7.0- 9.0 10.0-14.0 4.0- 5.5	18.0-21.0 7.0- 9.0 10.0-13.0 4.0- 5.5	18.0-22.0 7.0- 9.0 10.0-14.0 4.0- 5.5	18.0-23.0 7.0- 9.0 10.0-14.0 4.0- 5.5	18.0-22.5 7.0- 9.0 10.0-14.0 4.0- 5.5	18.0-23.0 7.0- 9.0 10.0-14.0 4.0- 5.0	18.5-24.5 7.0- 9.0 10.0-15.5 4.0- 6.0	18.0-23.0 7.0- 9.0 10.0-14.0 4.0- 6.0	18.0-23.0 7.0- 9.0 10.0-14.0 4.0- 6.0
				Hind legs		Toad-shape faintly visible	distinct	Toad-shape noticeable						
June 16			Hind legs											
June 18	18.0-23.0 7.0- 9.0 10.0-14.0 4.0- 6.0	18.0-23.0 7.0- 9.0 10.0-14.0 4.0- 6.0	17.0-21.5 7.0- 9.0 10.0-12.5 4.0- 5.5	17.0-21.5 7.0- 9.0 10.0-12.5 4.0- 5.5	17.0-21.5 7.0- 9.0 10.0-12.0 4.0- 5.5	17.0-22.5 7.0- 9.0 10.0-14.0 4.0- 5.5	17.0-21.0 7.0- 8.5 10.0-13.0 4.0- 5.0	17.0-21.0 7.0- 9.0 10.0-13.0 4.0- 5.0	16.5-21.5 6.0- 8.5 10.0-13.0 4.0- 5.0	17.5-22.0 7.0- 9.0 10.0-14.0 4.0- 5.0	17.5-22.0 7.0- 9.0 10.0-13.0 4.0- 5.0	18.5-24.5 7.0- 9.0 10.0-15.5 4.0- 6.0	18.0-23.0 7.0- 9.0 10.0-14.0 4.0- 6.0	18.0-23.0 7.0- 9.0 10.0-14.0 4.0- 6.0
June 19					Toad-shape distinct		Fore legs	Many dying						
June 20				Toad-shape								A few have hind leg buds		
June 21			Toad-shape											Hind leg buds
June 23							First metamorphosis; typical toad, no tail; length 6.5 breadth 3						Hind leg buds	

June 24		Hind leg buds												
June 25	18.5 23.0 7.0-9.0 10.0 14.0 4.0 6.0	18.0 24.0 7.0-9.5 10.0 14.5 4.0 6.0	17.5 22.0 7.5-9.0 10.0 13.0 4.0 6.0	17.0 22.0 7.0-9.0 10.0 13.0 4.0 6.0	17.0 22.0 7.0-9.0 10.0 13.0 4.0 5.5	17.0 22.0 7.0-9.0 10.0 14.0 4.0 5.5	17.0 21.0 7.0-8.5 10.0 13.0 4.0 5.0	15.5 20.0 6.0-8.0 9.5 12.0 4.0 4.5	15.0 20.5 5.5-8.0 8.5 12.0 3.5 5.0	15.0 21.0 5.5-8.0 9.5 13.5 3.5 4.5	16.0 20.5 6.0-8.0 9.5 12.5 4.0 4.5	20.0 24.5 7.0-9.5 10.0 15.0 4.0 6.0	19.0 24.0 7.0-10.0 12.0 14.0 4.0 6.0	20.0 24.0 7.0-9.5 12.0 14.5 4.0 6.0
	Hind leg buds		Leg buds 2.0		Legs 2.5	Legs 3.5	Legs 3.5 Toes good	Legs 3.5-4 Toes very good	Toes very good	Legs 3-4				
June 26										Fore legs				Hind legs
June 27									Fore legs			Hind legs		
June 29		Hind legs	Seem to recover from the thyroid influence					Fore legs					Hind legs	
July 1										First metamorphoses				
July 2									First metamorphosis					
July 5								First metamorphosis						
July 7														Fore legs
July 10		Fore legs											Fore legs	
July 11	18.5 23.0 7.0-9.0 10.0 14.0 4.0 6.0	18.0 24.0 7.0-9.5 10.0 14.5 4.0 6.0	18.5 22.0 7.5-9.0 11.0 13.5 4.0 6.0	18.0 22.5 7.0-9.0 11.0 13.5 4.0 6.0	17.5 22.0 7.0-9.0 10.5 13.0 4.0 5.5	17.0 22.5 7.0-9.0 10.0 14.0 4.0 5.5	17.0 21.0 7.0-8.5 10.0 13.0 4.0 5.0	10.0 7.0 3.0 3.5	11.0 18.0 5.5-6.5 5.5 11.5 3.5-4.5	14.0 18.0 5.5-7.0 8.5 11.0 3.5 4.5	16.0 18.0 5.5-7.0 8.5 11.0 3.5 4.5	20.0 25.0 7.0-10.0 10.0 15.0 4.0-6.0	20.0-25.0 7.0-10.0 13.0 15.0 4.0 6.0	20.0-25.0 7.5-10.5 12.5 14.5 4.0-6.0
July 16														First metamorphosis
July 18		First metamorphosis						Last metamorphosis					First metamorphosis	
July 22									Last metamorphosis					
July 23										Last metamorphosis				

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[1] When four measurements are given, the first refers to the entire length, second to length of body, third to length of tail, fourth to breadth of body.

The main interest of Experiment X lies in the results following the mixed feedings of two kinds of animal tissues or animal tissue and plants.

It will be seen from the notes that, whenever thyroid tissue was one of the two foods it exerted an accelerating influence on development. The four groups fed on thyroid-thymus (fig. 9 *h*), thyroid-muscle (fig. 9 *f*, 9 *n*), thyroid-hypophysis (fig. 9 *i*, 9 *p*) and thyroid-plants (fig. 9 *g*, 9 *q*) developed their hind legs on the 5th day. The fore legs appeared in the third and fourth groups on the 21st day, in the second group on the 22d day and in the 1st group on the 24th day and the tadpoles of the several groups began to metamorphose in the same order. These facts indicate that the thymus was best able to counteract the thyroid, the hypophysis and plants least. It is peculiar, however, that the order of the last individual metamorphoses is exactly the reverse, the thyroid-thymus group completing the change first, the thyroid-plant group last. It is doubtful whether this has any connection with the fact that the greatest reduction in size was in the first group and gradually less down to the fourth.

We may now compare these thyroid-thymus results with those of Experiments VIII and IX. They are essentially alike, the thymus always being able to check the thyroid influence to some degree, but unable to suppress it entirely (fig. 2 *k*, 2 *m*, 6 *b*, 6 *d*, 9 *h*).

The remaining three groups, thymus-hypophysis (fig. 9 *i*, 9 *p*), thymus-plants (fig. 9 *k*, 9 *q*) and hypophysis-plants run much slower than the corresponding thyroid groups. Their hind legs appear from 16 to 19 days later than in the thyroid combination feedings.

The hypophysis-plant group leads the three, the thymus-plant group is a few days behind. This corresponds to the thyroid groups, where the hypophysis was less able to counteract the thyroid acceleration than was the thymus. In the thymus-hypophysis group (9 *l*, 9 *r*) two retarding factors combine, therefore the tadpoles fed on this diet never develop fore legs nor do they begin to metamorphose. Yet they are very big healthy specimens.

It is thus clear that, whenever thyroid forms a part of the diet, a precocious differentiation sets in; when thymus is added (thymus-thyroid, thymus-hypophysis, thymus-plants), the opposite influence is noticed.

One group (9 *m*) of Bufo tadpoles was starved to test again whether hunger might cause differentiation, but no differentiation was noted even 5 weeks after the start of the experiment.

Finally another group was fed on testicle. The testicle diet had not been fully tested before. The development of these tadpoles did not differ from the control and this group can therefore serve for a further comparison with the groups given a mixed diet.

Experiments were also begun on Triton alpestris and on Bufo vulgaris tadpoles which had a part of their tails amputated, but the time was too short to carry them out completely.

Feedings were started with Triton alpestris larvae on June 24. On July 12 the outer gills of the thyroid-fed ones had disappeared and the fore legs were well differentiated. The thymus-fed specimens still possessed their outer gills and had only small buds of their fore legs. The animals treated with other foods showed intermediate conditions.

Bufo vulgaris tadpoles of 19 to 23 mm in length, tails 10 to 14 mm. long, had 5 mm of their tails amputated on June 24. The next day separate groups were started on thyroid, thymus, muscle and vegetable diets. The thyroid group developed hind legs on July 3 (8th day) and fore legs on July 12 (17th day). The thymus and muscle groups developed hind legs on July 9 (14th day). Their measurements are given in table 15, page 468.

The thymus-fed ones had grown considerably by July 14 and regained their former average length, since they had almost completely regenerated their tails. The other groups had grown and regenerated much less, the thyroid least of all, while in turn it showed the fastest differentiation. These observations correspond with those of 1911. *Romeis* has studied extensively the

TABLE 15

	CONTROL VEGETABLE	THYROID	THYMUS	MUSCLE
	mm			
June 24	19.0–23.0	(14–19 mm after amputation)		
	7.0– 9.0			
	10.0–14.0	(5– 9 mm after amputation)		
	3.5– 5.5			
July 14	13.5–18.0	11.0–14.0	20.0–22.0	15.0–18.5
	7.0– 9.5	5.0– 6.5	9.0–10.5	7.5– 9.5
	6.0– 9.0	6.0– 8.0	11.0–12.0	7.5– 9.0
	4.0– 5.0	2.5– 4.0	5.0– 6.5	4.5– 5.5

influence of different diets on the regeneration of the tail in Rana esculenta tadpoles.

The experiments of 1911 and 1912 leave no doubt that the mammalian thyroid gland contains an *agens* which, when the gland is given as food, calls forth a rapid differentiation in a developing vertebrate organism. This differentiation may be brought about at any stage of development, before maturity is reached. The differentiation may therefore be highly precocious in cases where the treatment is begun on extremely young animals. Experiment I showed that tadpoles could be brought to the point of metamorphosis within 18 days after hatching, while normally they would require 10 to 12 weeks to reach such a stage. The results of this premature metamorphosis are perfect frogs of minute size, pygmy frogs, as the figures show (fig. 4 *o*, 4 *p* and others). The thyroid influence is very decided and there is no escape from it for any tadpole given a thyroid diet. All of the individuals, even if thousands be employed as was the case in several experiments, will react almost immediately to the thyroid stimulus, so that certain changes in their structure may be observed after 24 hours, when only one application of thyroid has been made. They will all react simultaneously so that any individual differences in development will become unobservable.

The experiments further bring out the fact that the time of reaction to the thyroid stimulus varies to a certain degree with the kind of food that had been given before the thyroid diet was started. There is also a difference in response between previously starved and fed tadpoles. The experiments with mixed diets showed that the accelerating influence of the thyroid could be checked to some extent and the rapid differentiation more or less retarded. However, there is no complete counteraction against the thyroid stimulus, so that sooner or later any tadpole, receiving thyroid after other or mixed with other diets, must respond to its influence.

Experiments were carried out to determine the least amount of thyroid food necessary to produce the typical reactions, and also to determine whether or not the tadpoles could recover from the thyroid shock if afterwards put on other food. A feeding of only 24 hours –that is to say, the thyroid was kept in the dish for about 24 hours, though the animals did not feed on it continuously —sufficed to cause a hastened differentiation. A feeding for three days was enough to give the fastest rate of differentiation, a rate that could not be increased by longer feedings.

When thyroid is applied too rapidly, the animals usually die very soon after the appearance of their fore limbs and the simultaneous reduction of their tails. By careful feedings at rather long intervals and in all not more than four times, the animals may be kept alive for several weeks. They will not undergo, however, any further changes, except perhaps a continued reduction of their tails, nor will they ever feed again. In 1911 I succeeded in bringing some tadpoles to a complete absorption of their tails and these thyroid frogs were kept alive on wet sand for from 2 to 4 days. In 1912 some Bufo tadpoles almost completely absorbed their tails under the thyroid treatment (fig. 9 *e*, 9 *n*), but could not be kept alive for more than 24 hours.

A recovery from the thyroid influence is extremely rare. Only 5 individuals out of 400 in one experiment and 6 in another were able to survive, and, although, they were never seen to feed, began slowly to grow again after a standstill of several weeks.

Not one of the many thousand tadpoles fed on thymus in the spring of 1912 could be brought to metamorphosis during the 15 weeks in which the animals were under observation, while tadpoles of the same set, though fed on other substances, metamorphosed before that time (fig. 4 *o*, *p*, liver). In some experiments the thymus fed tadpoles never succeeded in growing their hind extremities before the controls completely metamorphosed. Yet the thymus tadpoles would grow very rapidly, especially during the first weeks of the experiments.

The tadpoles feed on spleen behaved in very much the same way as the thymus-fed ones, though they always were somewhat ahead of the latter and did not counteract the thyroid feedings so strongly. Of the great number of spleen fed tadpoles also not one could be caused to metamorphose.

We must therefore conclude that the thymus and to some extent the spleen also, and probably the lymphatic organs, when given as food, will cause a rapid growth followed by a rather late differentiation or none at all.

These experiments with various foods, except thyroid, thymus and spleen, and the investigations of other workers show that the animals must reach a certain constant minimum size before the final metamorphosis can begin. There is on the other hand a constant assimilation of food and a gradual increase in body size up to a constant maximum, beyond which a normal animal may not pass without the beginning of the final differentiation and metamorphosis.

In the thyroid-fed tadpoles there is differentiation without growth, in the thymus- (and spleen-) fed tadpoles growth without differentiation. The experiments, therefore, emphasize the fact that in development we deal with two entirely separate factors, the factor of growth and the factor of differentiation.

The two naturally work simultaneously; but differentiation is not the result of growth, otherwise there could be no thyroid differentiation without growth. Nor must growth necessarily be followed by differentiation, as seen in the growing thymus tadpoles.

One might say that ordinary foods which are being assimilated bring with them the two factors of growth and differentiation. Then the assumption is necessary that the thyroid food lacks the power of causing growth, while the thymus and spleen lack the power of causing differentiation. But we can hardly assume that all the various kinds of foods an animal may take in, with the only exceptions of thyroid and thymus, contain these two factors in the proper proportions.

The factors for growth and differentiation can only be located within the organism itself.

We face the following propositions:

1. The thyroid has the power to excite differentiation, but it lacks the power to cause growth.

The thyroid calls forth differentiation, whether the animals be small or large, and without regard to the standard minimum size, necessary for the final change. It must possess an *agens* for stimulating differentiation which other foods do not possess. That the thyroid also possesses a power which prevents growth is not evident. The suppression of growth may merely be incidental, for rapid differentiation does not allow growth.

2. The thymus has the power to stimulate growth, but lacks the power to excite differentiation.

It has been stated above that tadpoles feeding on any food (except thyroid) reach a maximum size, and when this maximum is reached, differentiation begins independently. The thymus-fed tadpoles reach this maximum size and differentiation should set in of itself, even if the thymus lacked the necessary stimulus. But differentiation does not begin; therefore the thymus (and spleen) must exert an influence not possessed by the other foods which suppresses differentiation. That the thymus possesses the power to stimulate growth, is again not so evident, since the thymus growth may be merely the normal result of the intake of food. The thymus growth is rather rapid, but this may be attributed to the better nutritive qualities of the thymus tissue. The thymus-fed tadpoles may also grow beyond the normal as brought out in

1911, yet this again may be merely incidental. There is no beginning of differentiation in thymus-fed tadpoles which would check a further growth.

We can only say with reasonable safety:

1. The thyroid possesses a quality that stimulates differentiation, not contained in any other food used.[2]

2. The thymus (and spleen) possess a quality that suppresses differentiation not contained in any other food used.

Thus the thyroid and thymus must produce, or at least contain, agents which, when passed into developing organisms, in the one case stimulate, in the other suppress, differentiation. The production of such substances to be thrown into the circulation characterises the thyroid and thymus as glands with a positive internal secretion. That these two types of tissues may also be capable of performing the reverse action, viz. the elimination of certain substances from the circulation, as has been assumed especially for the thyroid, is neither demonstrated nor denied by these experiments.

The other glands used in the studies may or may not contain either the accelerating or the depressing power. However, the macroscopic differences in the rate of growth and differentiation of tadpoles fed on such glands and of the control animals are only slight and might easily be attributed to differences in the nutritive values of the various foods. The feeding experiments on tadpoles are, therefore, not likely to reveal these factors, if at all present in other glands, in a striking degree. Their study must be left to experiments of a different kind.

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[2] By 'differentiation' is meant merely the macroscopic changes, hind, forelimbs, metamorphosis. The microscopic differentiation will be discussed in a later paper.

LITERATURE CITED

Barfurth, D. 1887 a Versuche über die Verwandlung von Froschlarven. Arch. f. mikr. Anat., Bd. 29, p. 1.

1887 b Der Hunger als förderndes Prinzip in der Natur. Arch. f. mikr. Anat., Bd. 29, p. 28.

Basch, K. 1906 Beiträge zur Physiologie und Pathologie der Thymus. Jahrb. f. Kinderheilk., Bd. 64; 1908, Bd. 68.

Biedl, A. 1910 Innere Secretion, I. Auflage. Wien-Berlin.
1913 Innere Secretion, 2. Auflage. Wien-Berlin.

Gudernatsch, J. F. 1912 a Fütterungsversuche an Kaulquappen. Demonstr. Verh. Anat. Ges., Bd. 26. Vers. München.

1912 b Fütterungsversuche an Kaulquappen. Vorl. Mitteil. Centralbl. f. Physiol., Bd. 26, no. 7.

1912 c Feeding experiments on tadpoles.I. Arch. f. Entwickel.-Mech., Bd. 35, p. 457.

Hammar, I. A. 1910 Fünfzig Jahre Thymusforschung. Ergebn. d. Anat. u. Entwicklungsg., Bd. 19.

Lieben, S. Über die Wirkung von Extrakten chromaffinen Gewebes (Adrenalin) auf die Pigmentzellen. Centralbl. f. Physiol., Bd. 20.

Romeis, B. 1913 Der Einfluss verschiedenartiger Ernährung auf die Regeneration bei Kaulquappen (Rana esculenta) I. Arch. f. Entwicklungsmech., Bd. 37, p. 183.

Vincent, S. 1912 Internal secretion and the ductless glands. London.

All illustrations taken from living tadpoles. All figures in natural size except figures 10, *a* to *i* and 11, *a* to *f*.

PLATE 1

EXPLANATION OF FIGURES

1, a to ze Rana temporaria I. Experiment I. *a*, liver; *b*, spleen; *c*, thymus; *d*, muscle; *e*, ovary; *f*, adrenal cortex; *g*, adrenal medulla; *h*, brain; June 6, 1912. *i*, liver; *k*, liver-thyroid; *l*, spleen; *m*, spleen-thyroid; *n*, muscle; *o*, muscle-thyroid; *p*, ovary; *q*, ovary-thyroid; *r*, adrenal cortex; *s*, adrenal cortex-thyroid; *t*, adrenal medulla; *u*, adrenal medulla-thyroid; *v*, brain; *w*, brain-thyroid; June 26, 1912. In *k*, *m*, *o*, *q*, *s*, *u*, *w*, thyroid feeding had been started on June 17, 1912. *x*, liver; *y*, brain; *z*, spleen-thyroid; *za*, muscle-thyroid; *zb*, ovary-thyroid; *zc*, adrenal cortex-thyroid; *zd*, adrenal medulla-thyroid; *ze*, brain-thyroid, July 6, 1912. In *z* to *ze*, thyroid feeding had been started on June 17, 1912.

2, a to n Rana temporaria II. Experiment IV. *a*, original size, June 4, 1912. *b*, control; *c*, thyroid-muscle, thyroid given 1 day; *d*, thyroid-muscle, thyroid given 3 days; *e*, thyroid-muscle, thyroid given 5 days; June 17, 1912. *f*, thyroid-plants; thyroid given 1 day; *g*, thyroid-plants, thyroid given 2 days; *h*, thyroid-plants, thyroid given 4 days; *i*, thyroid-plants, thyroid given 5 days; *k*, thyroid-thymus; *l*, thyroid emulsion; June 17, 1912. *m*, thyroid-thymus, *n*, thyroid-muscle; thryoid given 4 days; June 26.

3, a to n. Rana temporaria III. Experiment III. *a*, thymus; *b*, muscle; *c*, thymus-thyroid I; *d*, thymus-thyroid II; *e*, thymus-thyroid III; June 1, 1912. *f*, thymus-thyroid II; *g*, thymus-thyroid III; *h*, thymus-thyroid IV; June 17, 1912. *i*, thymus; *k*, thymus-thyroid V; *l*, muscle; *m*, muscle-thyroid; June 26, 1912. *n*, muscle-thyroid; July 6, 1912.

5, a to e Rana temporaria V to VII. Experiment VI. *a*, control; *b*, glandular hypophysis; June 26. *c*, control; *d*, glandular; *e*, neural hypophysis; July 6, 1912.

6, a to d Rana temporaria V to VII. Experiment IX. *a*, control; *b*, thymus-thyroid; June 17, 1912. *c*, control; *d*, thymus-thyroid; June 26, 1912.

THYROID EXPERIMENTS ON TADPOLES
J. F. GUDERNATSCH

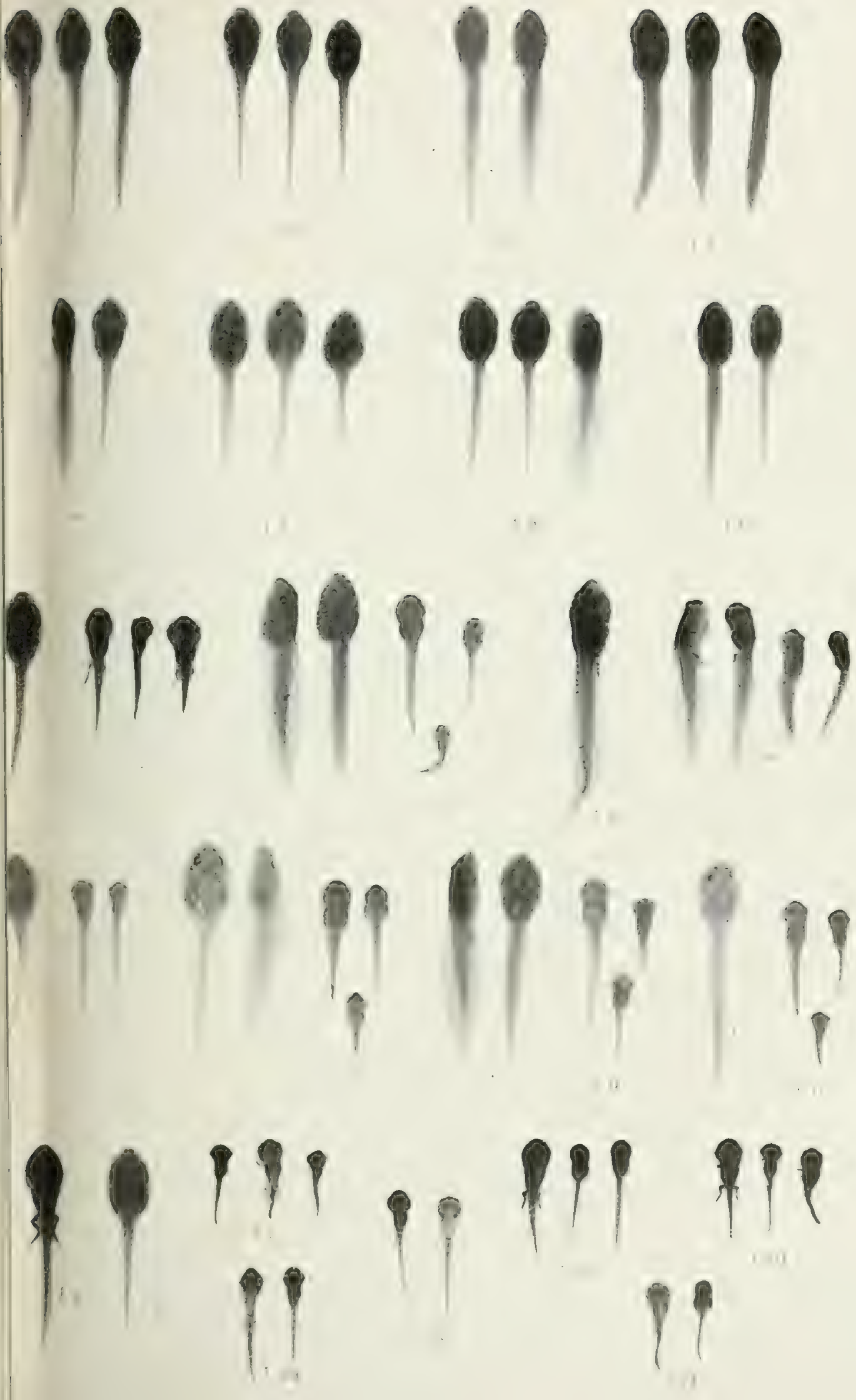
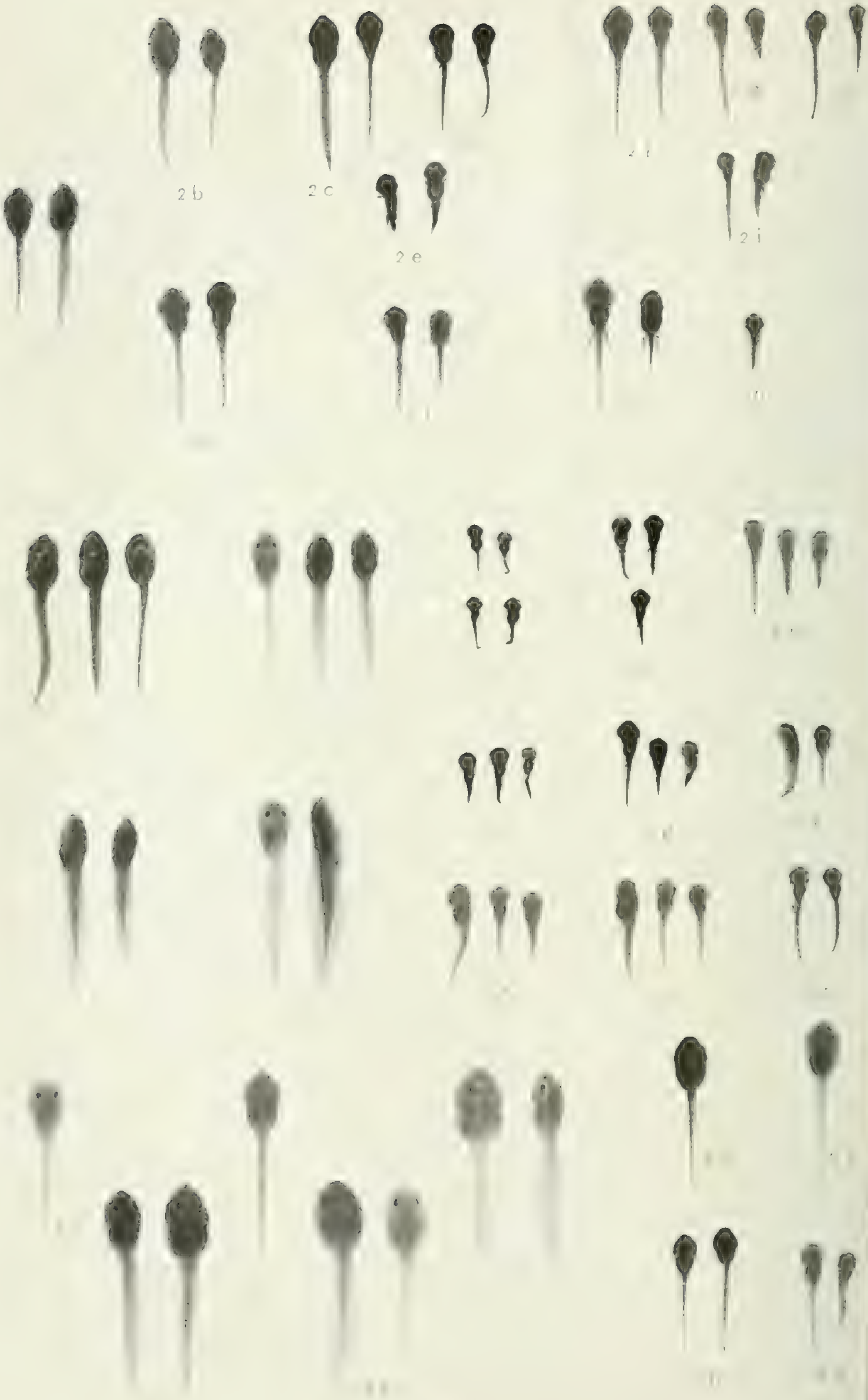
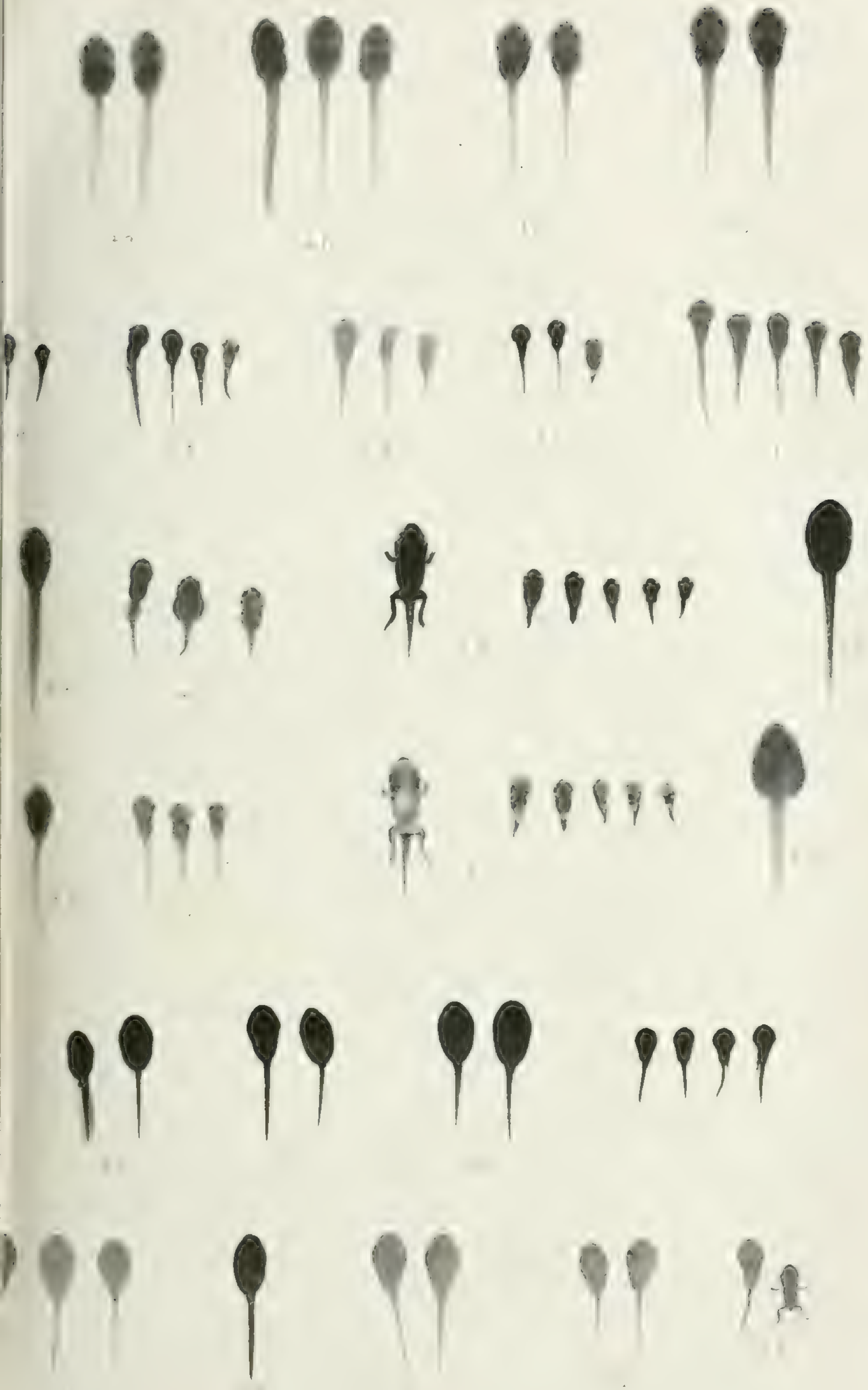
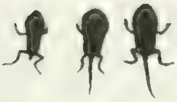
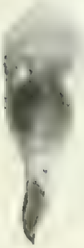


PLATE 1









THE TISSUE CULTURE AS A PHYSIOLOGICAL METHOD.

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The original necessity that led to the invention of the tissue culture method by Harrison developed out of researches aiming towards the proof of the origin of the nerve fiber. For the final proof of this problem it was necessary to completely isolate the growing nerve from other tissue cells and at the same time be able to observe its movements.

The working hypothesis was derived from observations made on growing nerve in many of his operated embryos. Harrison thought that if naked nerve fibers could traverse a blood clot interposed in their path within the body they could likewise grow in a similar clot outside the body. Previous work upon the survival of cells in vitro had conclusively shown that many body cells could live apart from the animal organism. The necessity lay in finding a technique suitable for using this fact as a means to promote the knowledge of growth. Ranvier, by a method identical to that of Harrison, observed the movements of leucocytes in vitro, and later Joly, repeating Ranvier's experiment, using the blood of Triton, noted division in many of these blood cells. These authors failed apparently to recognize a broader application of their method and it was forgotten.

Other methods, as the one devised by Leo Loeb in 1898, were not applicable to the study of the living tissues while in the culture and were therefore of little advantage over the study of prepared tissues from the animal body. The tissue was placed in a test tube and observation was impossible except through later study of sectioned and stained material. The primary object in growing tissue outside the animal body is in order that it may be microscopically observed in the living condition; this object was not possible by Loeb's method.

The ease of observation, the tissue isolation and the narrow and easily regulated environment of the culture which Harrison described and had shown to be fully applicable for the study of growth and differentiation of highly specialized tissues presented the more promising hopes. If it was possible to observe the growth and differentiation of the nerve fiber it was likely possible that many other cells could be studied and their conditions of function, growth and metabolism analyzed.

In the spring of 1910, through the kindness of Professor Harrison, I was allowed to take up a study of these cultures in his laboratory with the idea of more fully developing this method so as to make it applicable for a more careful physiological study of growth. The difficulties of such applications lay, as Harrison pointed out, in the lack of a suitable medium and tissue. It was primarily necessary to find a medium which would allow the use of a large number of tissues of other animals. The tissues must be dependent for nourishment upon the medium used.

Lymph, which Harrison had used, could be obtained from frogs but only in small quantities and its consistency varied widely at least in fibrin content, even in the different lymph sacs of the same individual. The medium which approached lymph most nearly in general consistency was blood plasma. This could be obtained from any animal and in quantities sufficient for the preparation of a large number of comparable cultures.

I found by the application of the well-known laws of blood coagulation that plasma could be readily prepared. Blood was collected directly from the heart, a vein or an artery through an oiled canula into a tube lined with a layer of paraffin and kept cold by surrounding it with ice. The chilled liquid blood was centrifugalized by placing the tubes in large centrifuge receptacles which were filled with salt, ice and water mixture. The supernatant plasma when free from cells was pipetted to a clean tube and preserved in the ice box. The plasma of frogs and chickens could be kept by such a method for days or months. Recently I have discarded the use of oil and paraffin. Clean glassware answers as well for the preparation of plasma from the blood of any animal and greater cleanliness is assured.

I continued this work with tissues of chick embryos. The embryos could be readily obtained and represented a warm-blooded animal. The tissue cells were nourished in nature by a vascular system. They thus differed from the tissues of frog embryos in not having a cellular yolk supply. Any extensive growth of such tissues when isolated in a culture must be dependant for nourishment upon food obtained from the media. The cells of the young embryo frogs studied by Harrison might, on the other hand, receive ample nourishment from the yolk contained within them.

The growth from pieces of the tissues of chick embryos was more extensive but essentially the same as Harrison had observed from pieces of frogs. It consisted in a survival and an extensive outwandering of cells into the medium about the tissue. I was able to confirm by a study of pieces of the neural tube all previous observations made by Harrison as well as to observe other forms of cell activity most necessary for increasing the possibilities of study and for making more conclusive the proof of life *in vitro.* Mitotic division, not observed in cultures of frog tissues, was most common in cells which wandered into the medium from the tissues of chick embryos. The cultivated pieces of the hearts of embryos functioned. Rhythmical beating continued for many days, apparently to the exhaustion of the media.

Carrel and I, during the winter of 1910 and 1911, tested the applicability of this method to the study of adult tissues. We showed that plasma could be readily prepared from all animals and that tissue of both adult and embryonic mammals survived and showed activity in hanging drop cultures where either plasma of isoplastic or homoplastic origin was used. Malignant tumors were tested and their activity fully established. The animals used were chickens, rats, mice, guinea-pigs, dogs, cats and man. Many other laboratory animals have been carefully tested by other observers. The growths included both the epithelial and connective tissue cells. These observations have been fully confirmed by the work of Lambert and Hanes, Loeb and Addison, Braus, Weil, Hada, Oppel and others.

The tissues of all animals grow luxuriantly, excepting human tissues. The reaction of normal human tissues, whether embryonic, adult, carcinomatous or sarcomatous, is practically the same. There is rapid and complete liquefaction of the plasma clot. The entire clot may go into solution within two or three hours. Small growths may be observed but they consist only of a few cells wandering out over the cover glass. I tried in recent experiments to obtain growths by repeatedly transplanting the tissue to fresh media.

This gave no result. At each transfer the medium was liquefied until death of the tissue was in evidence. The dead tissue did not liquefy the medium. The clots remained firm about it for several weeks.

Simplification and further possibilities for a more careful analysis of growth were introduced by the work of Schorer, M. R. and W. H. Lewis. Schorer showed that nerve cells of chick embryos could be grown in agar. M. R. and W. H. Lewis confirmed this work and demonstrated growths of the tissues of embryo chickens in many liquid media, such as sodium chloride, Ringer's solution, Locke's solution and serum. It was interesting to note that the tissue grew even in widely different concentrations of pure sodium chloride, varying from two and one-fourth to seventeen parts per thousand. No growth took place without sodium chloride. The growth was prolonged and increased by addition of calcium and potassium. Additions of maltose or dextrose, or protein decomposition products, increase proliferation; bouillon agar increased it still more, while the most luxuriant growths were obtained in plasma.

The growths in Locke's solution or more complex synthetic media were often quite extensive. Some few mitotic figures were observed in the stained preparations.

The growth that took place about tissues in a plasmatic clot was practically the same as that growth seen from various tissues into a wound of the animal body. It is upon this similarity that the normality of tissue growth *in vitro* has been based. Connective tissue is active and grows generally as isolated cells into the surrounding media. The cell is most often spindle-shaped although irregularly shaped cells are not uncommon. All tissue cells grow quite characteristically to their kind. Epithelium from skin remains together in sheets or membranes. Few cells, however, often become separated and wander away as isolated elements. During their wandering they show some amœboid-like movements. The group form of the cells generally remains characteristic of the various sources from which they are taken. Thyroid epithelium tends generally to form tubes rather than membranes. These tubes are readily and easily distinguished from the tubular growths of kidney or other organs. Nerve cells invariably give rise to axis cylinder processes, as Harrison and I have shown for embryos and Marinesco and Minea for the spinal ganglia of mammals. Blood cells and their close relatives from the spleen, bone marrow and lymph nodes wander out as isolated and very actively amœboid elements.

Reproduction has been observed in connective tissue cells, blood cells and epithelium of glands and skin. The most active reproduction is seen in cultures of malignant tumors and embryonic tissues.

The survival of all cells in these cultures depends upon their ability to react to the media. To reach a suitable environment for life and activity they must wander from the tissue into the areas of richer nutrient media. Adult striated muscle fails to show any extensive ability to move, while embryonic muscle shows both active amœboid movement and corresponding changes in the fibrin.

In a more recent article I have shown that the isolated heart muscle cells of the chick embryo not only migrate but they can divide as well as differentiate and beat rhythmically. A careful study of these cells has shown further that each of these various states of activity is associated with some definite change taking place or established in the fibrin clot of the culture. That such was the case was not only shown by direct observation but by the fact that the cell could be changed from one state into the other. By changing the environment of a functioning cell, it was observed to divide. If the daughter cell were again brought into suitable surroundings they differentiated and beat rhythmically. It was interesting to note that these cells could not only be kept living in these active states in a culture at body temperature but it was likewise possible to keep cells in an inactive state of life for as long as six months. Remove such inactive cells to a new and fresh medium; they wander and divide.

These observations and additions to the technique of tissue cultures opened a broader field of physiological study and pointed out the possibilities most suitable for study by the tissue culture method. It was possible to reproduce many of those kinds of cellular activity that are seen in the animal body. A full analysis of the conditions which are associated with movement, division and differentiation in a tissue culture would have direct application towards a solution of many problems of growth in the body as well as a fuller understanding of the coördination and regulation of these activities so as to produce a proper organ and body form.

Ruth in 1911 showed that wound healing could be imitated in culture. He found that if holes were cut in pieces of frog skin the wound, if not too large, would heal. This healing took place largely through movement of the epithelial cells. The fibrous edges remained inactive for a time, later they moved together passively. He reproduced the picture of primary healing without scar.

Harrison showed that the necessity of solid bodies for the movement of cells could be fully demonstrated by means of the tissue culture. He observed that cells placed in liquid media remained spherical and would not move. When these cells were brought in contact with a solid body they changed their shape and moved out along the side of the solid. Such cell movement took place in lymph and plasma cultures by the contact of the cell with the fibrin strands. In liquid media they could move over the free surface of the liquid, the surface of the cover glass or along strands of spider web placed in contact with them. Carrel and I noted that movements of cells in liquid media took place only along solid support, such as cotton and silk threads which were placed in contact with the cells. These experiments confirmed more fully earlier observations upon the necessity of solid support for movements of cells in a wound (Leo Loeb).

Loeb and Harrison chose to call these movements evidence of stereotropism.

Carrel and I in 1910 and 1911 observed an increase in the movement of cells after adding distilled water and tissue extract to the plasma which was used as a medium. Ruth noted an increase in the movement of the epithelial cells of frog skin in plasma diluted with distilled water.

Carrel in a more recent article has emphasized this fact, giving it as evidence of making wounds in the body heal in a few days or hours.

Before one could draw such conclusions it was necessary to study more completely the laws governing the movement of cells in a culture and to establish some definite means of control. Further, are conditions in a tissue culture comparable to those in the animal body? Before drawing conclusions as to the specificity of a given substance it was necessary to study for control the effect of other simple diluting substances occurring normally in a coagulum.

Repetitions of these experiments made it seem certain that this increase in the migration of cells with each proportional increase of the diluting agent added to the plasma was not in any great part dependant upon a direct action of the dilutant on the cell but was dependant upon the mechanical changes its addition produced in the clot. To make this more clear some of the conditions influencing the movement of cells in the simple plasma cultures will be discussed.

The rate and the extent of cell movement in a culture, one can assume, vary with the qualitative and the quantitative changes of the stimulus or stimuli which initiate and guide them. They vary, likewise, with changes in the metabolism of the cell.

Harrison had considered that the stimulus for movement of cells in a tissue culture was contact with solid bodies. He termed this stereotropism. As Jacques Loeb states, "Stereotropism is no real tropism, inasmuch as in this case no lines of force exist." When a cell is spread over a solid surface, such as a cover glass, it would not change its position as the result of this contact alone. Opposite points of contact of the cell would be equally irritated. Changes in the shape of cells and migration are manifestations of one and the same property of protoplasm, namely, movement. It is well known that cells such as an amœba respond by movement to many external forces or stimuli. The cells in a tissue culture respond in a similar manner. When a cell spreads from spherical to a spindle form or from a spindle to a spherical form without changing its position, one must assume that the stimulus is acting equally on opposite points of the cell. Movements from place to place are dependant upon a greater spreading in one direction than in the other or a retraction at one pole and a flowing out of the protoplasm at the other. To cause a change in position opposite sides of the cell must be affected differently.

I had come to assume, therefore, that the solid body, although necessary for the maintenance of the proper shape and the migration of the cell, was not the active initiating stimulus for the migration of cells in a tissue culture.

Evidence in favor of such a view is shown by the following observations: When a piece of connective tissue is placed in a medium giving uniform support on all sides, the cells move out only in paths leading them directly away from the tissue fragment. They radiate out on all sides like light waves from a central illuminating body. Cells moving from a piece of tissue placed in a liquid medium and surrounded by a tangle of cotton threads, follow only those threads which lead them out and away from the tissue mass or other cells. Cross-threads are never occupied except when they lead the cell away from a mass of other cells.

It is well known that cells placed in fresh plasma or plasma liquefied by a few days of the growth of a tissue tend to send out processes, spread, and flatten over the surface of the supporting substance. As the medium about these cells later becomes exhausted and charged with acid waste products, the cells draw in their processes and become spherical.

When a piece of tissue is placed in the center of a drop of medium, an active metabolism of its cells causes rapid changes in that part of the medium which lies close in and about it. Acid can be demonstrated in the center of such a fragment even after a few hours of incubation.

Following such a local change in the medium, diffusion currents result. The waste substances within the tissue mass diffuse out into the surrounding areas of fresh medium. The fresh medium without streams into the tissue mass. The border cells of the tissue fragment are thus bathed on one side by a stream of acid waste products and on the other side by a stream of fresh medium.

The proximal pole of the cell is, therefore, continuously stimulated to draw in its processes, or at least its spreading is hindered, while the distal pole, on the other hand, is continuously stimulated to move outwards. The cell thus moves out and away from the tissue mass.

According to such a theory one must assume that the migration of a cell in a tissue culture is dependant upon changes brought about locally in the medium of a culture through the activity of the cell itself and neighboring cells. When a single cell is placed in a drop of medium, products of its metabolism are formed equally on all sides of the cell. Such a cell changes its shape but not its position. Place two cells together, they move slowly apart.

No better illustration of the dependance of one cell on another for the stimulus for migration can be given than through a study of the variations in the extent of migration of cells from pieces of tissue of different sizes. As illustrated in figure 1, the migration is greater away from the large than the small piece of tissue. The migration can likewise be shown to be greater away from a cellular than a fibrous piece of a given tissue. The greatest migration of cells has been found to be away from a cellular piece of a given tissue of 1 mm. in diameter or the largest piece of tissue in which all the cells remain active.

For the cells to react to these stimuli and produce them they must be well supplied with oxygen. Oxygen from the air diffuses into solid clots to a depth of 0.5 to 0.7 mm. in quantities sufficient to preserve the oxy-hæmoglobin color in red cells. A sharp line of demarcation between the oxy-hæmoglobin and reduced hæmoglobin remains at this depth even after many days exposure.

As the red cells suffer reduction of their hæmoglobin, other types of cells suffer, in layers of plasma thicker than 0.5 to 0.7 mm., hindrances in their movement and their metabolism.

It has been found that the greatest movement of spleen cells is in the thin layers of plasma, 0.2 to 0.3 mm. in thickness, and away from a piece of spleen 1 to 1.5 mm. in diameter. The surface area covered by cells about similar pieces of spleen decreases with each increase in the thickness of the layer of plasma up to certain limits. Above these limits of thickness (a layer 1 to 1.6 mm. in thickness) the growth reaches a constant minimum, Fig. 2.

The cells of pieces of heart muscle or skin of this size do not grow so actively in the very thin layer of medium. The greatest movement of the connective tissue and the epithelial cells occurs in layers of plasma of 0.5 to 0.7 mm. in thickness. The cells grow differently than do the cell from the spleen and suffer in the thinner drops apparently from a lack of food supply. Curves 1 and 2.

The cause of the increase in the migration of cells in the thin layers of the plasma I have assumed to be, first, the better oxygen supply to the tissue fragment and the migrating cells. The increase in the supply of oxygen to the tissue fragment is associated with an increase of the metabolism of its cells and a greater production of the repelling substances. Second, the repelling substances diffuse in greater concentration into the thin restricted areas of the medium; and, further, the cells migrating into the thin layers move in a single plane so that each cell is followed by a continuous line of actively metabolising cells.

The utilization of these facts and a careful technic has made it possible to obtain a constant type of growth from a given tissue into a plasma clot.

After repeating and controlling those experiments upon movement of cells in plasma diluted with tissue extracts or water, I found that a similar increase in the movement was noted after diluting the plasma with isotonic NaCl solution, Ringer's solution, Locke's solution, or serum.

This increase in the movement of cells after using serum as a dilutant suggested strongly that the decrease in the fibrin was the important factor. Plasma clots can be assumed to be composed of two constituents, fibrin and serum.

It is well known that fibrin clots, especially when they contain tissue cells, contract upon standing. The solid clot becomes smaller and the serum is squeezed out. The size of the contracted mass of fibrin is proportional to the quantity of fibrin present.

The same retraction of the clot occurs in a culture. The fibrin retracts against the cover glass. The serum drains off from the under free surface of the hanging drop. When pieces of tissue of similar size are planted in equally thick drops of plasma and plasma diluted with some liquid substance, the thickness of the solid medium varies in the different cultures after a few hours incubation.

As shown in figure 3, there is with each increase in the quantity of dilutant added to the plasma an increase in the area covered by cells about pieces of spleen of equal size. Comparison of figure 2 with figure 3 shows further that the area covered by wandering cells about the fragments of spleen placed in diluted plasma is similar to the area covered by cells about fragments of spleen placed in equally thick layers of contracted pure plasma.

This same relation between the thickness of the layer of medium and the migration of cells is seen in the study of movement of cells from fragments of heart muscle and skin placed in plasma diluted with various substances.

When the fibrin clots fail to contract, this definite type of increase of the movement of the cells is not noted in the diluted plasma. Spleen cells may show a slight increase in their movements in these less dense clots but the connective tissue cells suffer little more than an alteration in their arrangement.

The increase in the movement of cells in plasma after the addition of water, isotonic NaCl, Ringer's solution, Locke's solution, tissue extracts, or serum is in large part the result of mechanical changes occurring in the clot following a decrease in its fibrin content. Individual variations from the use of one or the other of these substances are small. Tissues planted in plasma diluted with water suffer early death, apparently, from hypotonicity. Isotonic NaCl activates movement somewhat more than Ringer's or Locke's solution. The cells survive longer in plasma diluted with tissue extract or serum than salt solutions. They die apparently in the plasma diluted with salt solutions from an early exhaustion of food materials.

It is questionable from the data at hand whether any great effect would be produced by the use of these substances in the body where the source of the oxygen and the mechanical conditions surrounding the coagulum are different.

It was little hoped that the tissue culture would have an important application for the study of the factors concerned in cell division. Its important use for the study of this problem seemed limited to the study of a few cells, such as the abnormal mitoses in tumors. Lambert has been able to observe abnormal mitoses in tumor cells growing in a culture.

It has been of interest to me during the last year to notice that many cells dividing in this particular environment show amœboid-like movements. These movements were not different in appearance from the amœboid movements of locomotion. There was, however, a difference in the source of the stimuli which initiated them. The amœboid movements of locomotion were apparently always associated with external factors while those of cell division were associated with changes taking place within the cell.

Many authors have figured amœboid processes in dividing cells, but no one has apparently called attention to the relation of the processes to a surface tension theory of division, nor studied their relations to nuclear changes in the cell.

Quincke held that amœboid movements were the result of surface tension changes along the wall of the cell. He assumed that the cell was surrounded by a fatty membrane and behaves like an oil droplet. When a drop of rancid oil suspended in water was touched at a point on its surface with a piece of sodium hydrate, which caused a decrease in its surface tension, this part of the drop bulged and gave rise to a pseudopode-like process. Bütschli extended and confirmed Quincke's results. He also assumed that cell division was a surface tension phenomenon. The cleavage furrow represented an area of greater surface tension than the remaining part of the cell. McClendon has added much evidence in favor of such a view. McClendon's oil droplet model of cell division represents very completely the conditions as I have observed them. He suspended a drop of rancid oil and chloroform between water and salt solution. By means of two pipettes equal quantities of sodium hydrate were allowed to flow simultaneously against two opposite points in the drop. The surface tension was lowered and the drop bulged at these points. To compensate for the bulging, constriction with following cleavage took place along the circumference of the drop midway between these points.

The changes in form taking place in the cell of a culture during mitosis are of two kinds. Primary with the onset of nuclear division the cell changes from a spindle shape to an oval or spherical mass of protoplasm, whose center is occupied by the nucleus. Later, after separation of the two groups of chromosomes, the cell elongates and cleaves at its equator. Following carefully the change in the shape of a cell from a flattened spindle to a sphere or an oval, it was found that this change often occurred as an active outward movement of that part of the cell wall nearest the nucleus.

The more distant end processes were apparently drawn inwards in compensation. Movements ceased in the small cells as soon as the walls were equally distant on every side of the nuclear spindle. Later in division, as the nuclear halves separated more and more from each other, they again came close to the wall at the poles of the cell. Following this approach, amœboid movements were seen at these parts of the cell. The cell slowly elongated with each movement, finally constricting at the equator.

That this movement was associated with the nuclear halves was more clearly shown by the study of cells whose early movements were hindered so that the dividing nucleus was excentrically placed. In these cases one line of chromosomes during its movements often came directly in contact with the edge of the cell. Following this contact an explosive outward movement at this part of the wall of the cell generally occurred.

One of the long disputed questions in physiology is that concerning the cause of the heart beat. Haller in 1757 taught that the heart was an organ independent of the nervous system. Remak in 1848, through the discovery of nerves in the frog's heart, naturally took the view that rhythm was initiated by the nervous system. Later the discovery of ganglion cells within the heart muscle strengthened this idea. In the latter part of the century the opposing view, that the heart was an automatic organ, was again raised. The difficulty for a proof of automaticity lay in a failure to completely rule out the possibility of existing ganglion cells. The neurogenic school offset all arguments as advanced by the adherents to the myogenic theory, by constantly holding up the fact that there was a possibility of undemonstrated ganglion cells in the young chick hearts and in the isolated and beating pieces of heart muscle studied by Gaskell and Engelman.

By the use of this method of tissue isolation I have found it possible to attack this problem and to prove more conclusively the automaticity of the heart muscle cell. Heart muscle cells completely isolated from all other tissue cells or possible nerve connections have been observed to beat rhythmically. The cells were taken from the hearts of fourteen and eighteen-day chick embryos—hearts which in the body are richly supplied with nerves. The isolated cells beat with a perfectly normal rhythm and with a rate and force similar to the heart in the embryo or pieces of heart muscle isolated in a culture.

The tissue culture is of use in studying problems of cell metabolism. I have found that the accumulation of fat in the cytoplasm of the cells of a tissue culture varies with changes in environment similar to cells in the body. These accumulations have been considered for most part infiltrations and not fatty degenerations. As Leathes expresses it, it is an inability on the part of the cell to burn the normal quantity of fat brought to it. It synthesizes and stores it up. In the cells of a tissue culture, fat accumulations vary with the quantity of fat in the medium and with the general state of metabolism of the cell. Cells in an environment perfect for active growth do not contain fat or lose the fat they have synthesized. Any disturbance of the metabolism of any cell in this same environment is associated with an increase in fat in the cytoplasm of the cell.

By these brief illustrations I have tried to show you not only some facts that have been brought out by the tissue culture method but to illustrate how this method is applicable for the study of physiological problems of growth.

EXPLANATION OF FIGURES.

Fig. 1. A camera lucida drawing showing the variation in the migration of cells away from a large and a small piece of spleen.

Fig. 2. Curve 1 shows the variations in the migration of cells away from similar pieces of heart muscle into layers of pure plasma of different thicknesses. Curve 2 shows the variations in the migration of cells away from similar pieces of skin into layers of pure plasma of different thicknesses.

Fig. 3. Camera lucida drawings showing the variations in the migration of cells away from similar fragments of spleen (a, b, c, d, e, f) into layers of pure plasma of different thicknesses.

Fig. 4. Camera lucida drawings showing the variations in the migration of cells away from similar pieces of spleen (a, b, c, d, e, f) into equally thick layers of pure plasma and plasma to which various quantities of serum had been added.

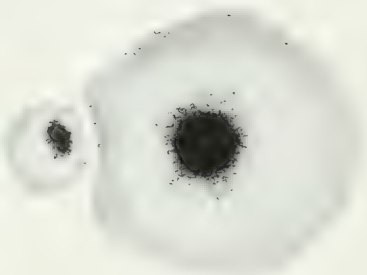
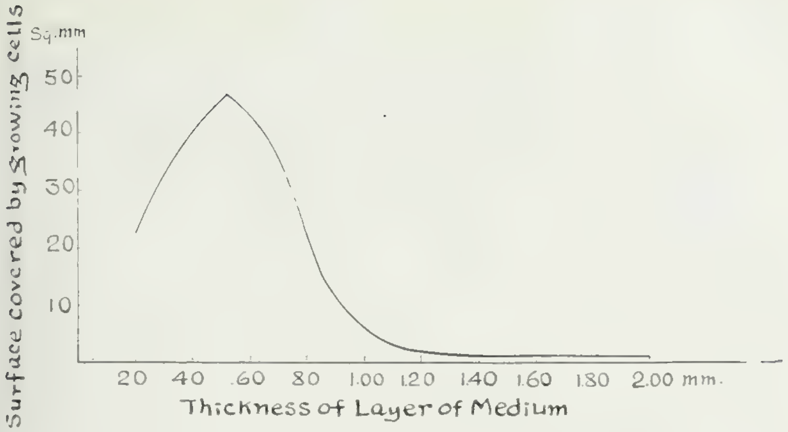
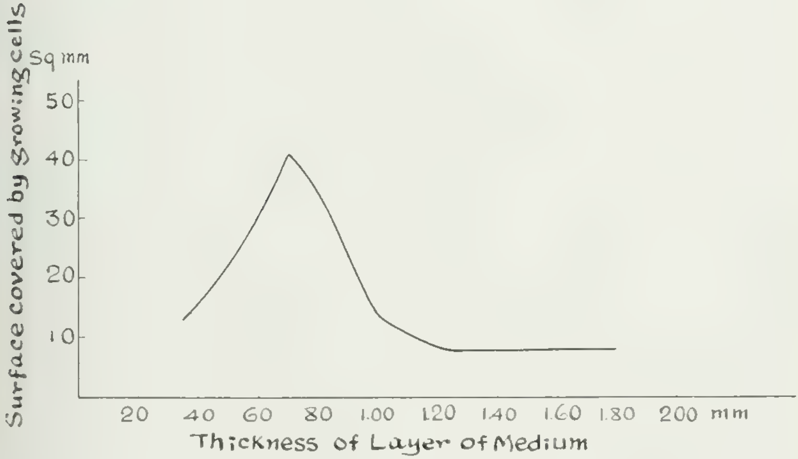


Fig. 1. A camera lucida drawing showing the variation in the migration of cells away from a large and a small piece of spleen.

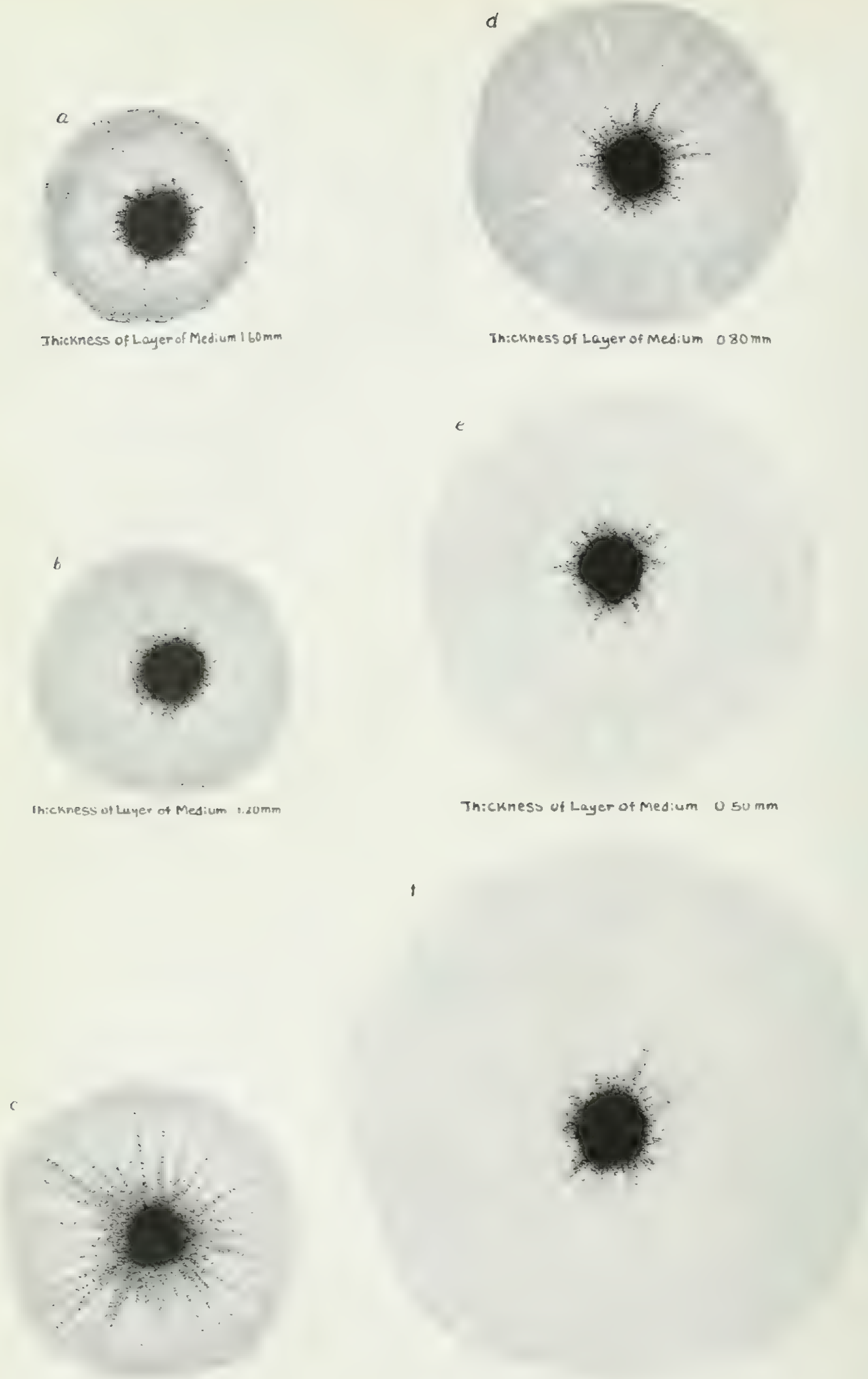


Curve 1 shows the variations in the migration of cells away from similar pieces of heart muscle into layers of pure plasma of different thicknesses.



Curve 2 shows the variations in the migration of cells away from similar pieces of skin into layers of pure plasma of different thicknesses.

Fig. 2.



Thickness of Layer of Medium 1.60 mm

Thickness of Layer of Medium 1.20 mm

Thickness of Layer of Medium 1.00 mm

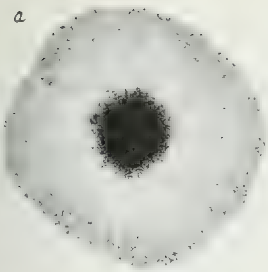
Thickness of Layer of Medium 0.80 mm

Thickness of Layer of Medium 0.50 mm

Thickness of Layer of Medium 0.30 mm

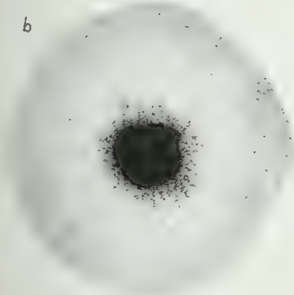
Fig 3.

a



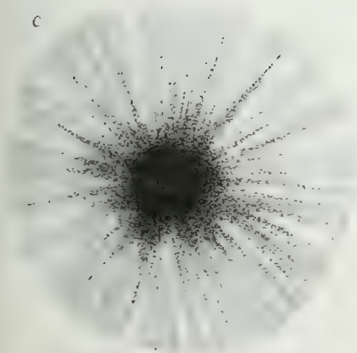
Pure Plasma
Thickness after 48 hours 1.3 mm

b



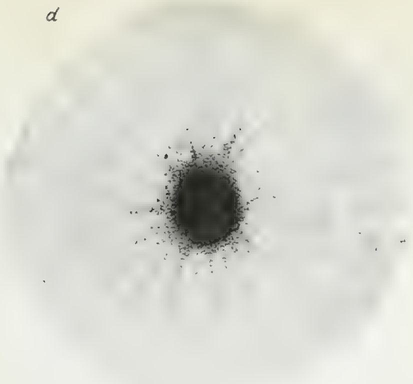
8 vol. of Pure Plasma + 2 vol of Serum
Thickness after 48 hours 1.25 mm

c



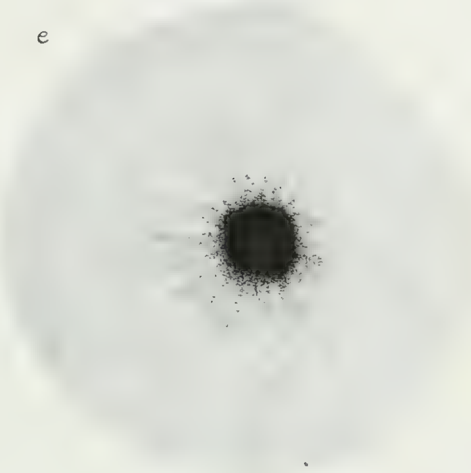
6 vol. Pure Plasma + 4 vol. of Serum
Thickness after 48 hours 1.2 mm

d



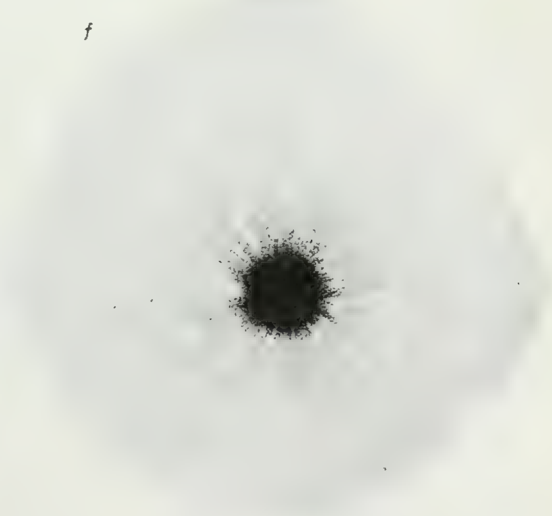
4 vol Pure Plasma + 6 vol. of Serum
Thickness after 48 hours 1.00 mm

e



2 vol Pure Plasma + 8 vol. of Serum
Thickness after 48 hours 0.60 mm

f



1 vol. Pure Plasma + 9 vol of Serum
Thickness after 48 hours 0.35 mm

Fig. 4.

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WOUND HEALING *IN VITRO*

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As I pointed out in an early communication the lymph and plasma methods of tissue cultivation are modelled after the process of wound healing in the body. Consequently, it seemed logical to assume that an analysis of the factors concerned with the growth of cells in a culture would aid in a better understanding of the process of repair in the body.

A large number of experiments have been performed by several observers including myself for the purpose of determining conditions more favorable for the growth of cells in a culture, hoping through such studies to better the methods for the treatment of wounds. No one has made, however, up to the present time a careful study of the conditions associated with the growth of cells in the control cultures. Such a study became important not only for the light it might throw upon the mechanism of healing in the body but also from the necessity of establishing some definite means of judging the results of further experimentation.

In the present paper I wish to present some observations upon the movement of epithelial cells and, further, to point out some of the changes that occur in the environment of cells during their migration and to show how cellular movement in a culture finds the most satisfactory explanation by the surface tension theory.

As a basis for the first series of experiments I shall consider more carefully some observations made by Ruth in 1910 and 1911 upon the cicatrization of frog skin *in vitro*.

Ruth found that if small holes were cut in pieces of frog skin and these pieces were planted in a drop of plasma the wounds, if not too large, would heal. This healing took place largely from the epithelial layer, the fibrous tissue portions showing no evidence of cellular activity. The epithelial cells moved out from all sides of the wound either as single cells or sheets of cells and eventually covered the wound. Later, the fibrous tissue portions, previously remaining inactive, moved slowly and apparently passively together.

More careful analyses of these wounds have shown that the healing of the epithelial layer was perfect in many cases; no marring remained to mark the site of the original cut. The interesting features to be studied were, first, what force attracted or brought together the inactive fibrous tissue portions, and, secondly, how did the epithelial cells restore themselves so completely to their former shape and arrangement? It seemed evident that the process must have taken place through a form of cellular movement for no cell division was seen in these specimens. That this is true is shown by the following experiment:

A strip of frog skin 5 mm. in length and 1.5 mm. in breadth was cut into five pieces of nearly equal size and placed in a row in a drop of plasma. The distance between each piece was from 0.2 to 1.2 mm. After a few hours liquefaction of the fibrin was noticed by the formation of a small cavity near the tissue border. The cavity slowly increased in size and opened on one or the other surfaces of the clot. Subsequent to the formation of the cavity the germinal layer of the skin moved out as a continuous sheet of cells over the inner surface of the cavity and out onto the surface of the clot. Liquefaction of the clot continued along the sides of the membrane until a deep furrow was formed in the coagulum. The outer border cells of the membrane continued to move outwards clinging to the fibrin at the edge of the furrow while the intermediate cells became free from the fibrin surface. The membrane thus became stretched through an open liquid cavity between the tissue on one side and the solid clot without.

The membrane increased in surface area through a continuous outward movement of its border cells and a stretching more and more of the cells within it. The membrane at first several cells in thickness was soon reduced to a single layer of flattened cells which were elongated in the direction of the movement.

Eventually adjacent portions about each piece met and fused with the membrane of its neighbor. The fibrin about the pieces underwent complete dissolution. The separate pieces then swung in an open liquid cavity by a continuous membrane of cells which were attached to the fibrin wall without.

Growth ceased after a few hours. The cells remained inactive for a time when they finally let loose their fibrin attachment, retracted and took their former position within the tissue fragments. The retraction of the membrane between the tissue fragments brought the fibrous tissue portions together. The strip appeared again the same as before it had been cut into pieces, except for a poor cementing together of the fibrous tissue portions.

This process of healing is the result of an active movement of outer border cells of the epithelial layer and a stretching or a staining of the intermediate cells in the layer. With the release of the force exerted at either end the elastic membrane returns immediately to its former shape.

In order for such a membrane to form, a system of forces working in opposite directions must exist. The tissue must be held firmly on one side and the clot must have sufficient firmness to overcome the stress of the cells moving over its surface. Often one notices that the fibrin is changed into coarse bands as the result of the pull of the cell upon it. In other cases when the fibrin clot finds no attachment to rigid bodies without it is pulled in mass toward the tissue and eventually entirely dissolved.

The same failure of the formation of a membrane is interestingly shown when the tissue fragment is unsupported. A piece of tissue was placed in a drop of fibrin and after clotting had intervened it was cut loose from the clot on all sides except one. This piece of skin showed only a narrow membrane of cells on its attached side. The whole piece of skin on the other hand showed active movement. It traveled several millimeters across the culture, resembling a machine plowing its way through the solid coagulum.

The same phenomenon is seen in wounds where the edges are loosely connected with the underlying or surrounding tissue. Linear wounds heal completely often by a continuous and slow moving together of the walls on the two sides.

The walls of round or rectangular wounds continue to move together by such a process until their edges become more compact and resist the stress of the membrane which now stretches to bridge the gap. Later, an active cellular proliferation increases the number of cells in the membrane allowing them to return to their former shape and thickness.

What has been found true in a tissue culture for the movement of the epithelial cells is likewise true for connective tissue cells. Wounds made in pieces of chicken skin may contract to a fraction of their original size during the first few hours after the preparation of the culture. In many cases activity was seen only in the cellular connective tissue layer. The cells during this contraction were just visible along the border of the tissue. The distal poles of the cells were in contact with the fibrin while the proximal poles remained attached to the cells within the fragment.

That the work necessary to stretch the membrane or move the piece of skin was the result of a decrease in surface tension along the outer edge of the cell membrane became hypothetical through a further study of the factors concerned with such movement. In many of the cultures the outer border cells show evidence of an active amœboid movement. Rounded or pointed processes may be seen to move outward and later to be slowly withdrawn. In other cases the membrane may move a considerable distance maintaining throughout a perfectly uniform straight border. The same is likewise true of connective tissue cells. Frequently

I have seen connective tissue cells move a considerable distance without showing visible changes in their contour or the arrangement of the fat droplets contained within them.

Leo Loeb noted that the epithelial cells in a wound move only when in contact with solid bodies. Harrison analysed this property of cells by the aid of the tissue culture. Harrison found that cells placed in a liquid became spherical and did not move unless they came in contact with some solid surface. In plasma cultures they moved along the fibrin strands, in liquid medium movement was observed along the surface of the cover glass, along strands of spider web or over the free surface of the liquid.

Leo Loeb and Harrison chose to call these movements evidences of stereotropism; *i. e.,* a turning of the cell towards a stimulus given by a solid body—a movement which results from the close apposition of the cell to the stimulating body.

If such were the case, the cell would migrate independent of its neighbors and dependent alone on the contact stimulus. When single epithelial or connective tissue cells are placed in a drop of plasma of uniform consistency, they spread out over its surface but they have never been seen to change their position.

When two cells, on the other hand, are placed close together, they move slowly apart. Cells migrate farther away from a large than a small piece of tissue; farther away from a cellular than a fibrous piece of tissue.

A careful survey of the above observations shows that the changes in the shape of cells as well as their migration find a close analogy to surface tension changes in many other liquid substances—an analogy made by Quinke as early as 1867 in explanation of protoplasmic movement. Quinke assumed that the cell responded much the same as a drop of oil. A drop of oil suspended in water takes a spherical form. A similar drop placed on the surface of water flattens out. The surface tension of the drop immersed in water is equal on all sides. The drop placed on the surface of the water comes, on the other hand, under the influence of three surface tensions: the air-oil surface tension, the oil-water surface tension, and the water-air surface tension.

The first two tend to round off the drop while the last tends to draw it out. Since the water-air tension is greater than the sum of the other two, the drop continues to spread until it covers the entire surface of the water.

The cell through an active metabolism produces continuously changes in the surrounding medium. Food materials are used up and acid waste products are liberated.

It is well known that when fatty acids are brought into solution in water they condense at its surface and cause a lowering of surface tension. When cells are placed in fresh medium they spread at its surface. Subsequent to an active growth and an exhaustion of the medium they become spherical. Remove these cells quickly to fresh medium they spread again at its surface.

These observations give a ready explanation of the migration of cells in a tissue culture. When a fragment of tissue containing many cells is placed in a culture medium an active metabolism of the cells in the midst of the fragment causes rapid changes in the small amount of medium which bathes them. Acid can be demonstrated in the center of the fragment even a few hours after the preparation of the culture. Such a local change results in diffusion currents. The border cells of the tissue must be bathed on one side by a stream of acid waste products and on the other side by an incoming stream of fresh medium. The cell is thus supported by a surface film having a lower surface tension at its proximal than distal part. The cell must then be carried out and away from the tissue mass.

If a piece of connective tissue or spleen be placed in a medium presenting a uniformly free surface on all sides of the tissue fragment, the cells migrate out along such lines of diffusion. They radiate out on all sides of the tissue like light waves from an illuminating body.

There is found in all these cultures a close relation between the rate and extent of cellular migration and the quantity of acid produced. The greatest movement of cells is seen always during the height of the acid production. Later in the history of the culture when less and less acid is liberated in the center of the fragment there is a progressive decrease in the movement of the cells.

Further evidence in support of the surface tension theory is seen in the movement of red blood cells, tissue debris and fat droplets which frequently accumulate in the medium about the tissue fragment. With the onset of activity in the center of the tissue mass these substances move out into the surrounding medium. They follow the same course to be later followed by the moving cells.

I have not presented this theory of surface tension as new. I have given it here in some detail since other theories have been advanced and it seems to give the most plausible explanation of the facts now known about the movement of tissue cells in a culture. It is hoped by the utilization of this theory and these facts that a further and more complete analysis of wound healing can be made.

(Lantern slides)

[Saturday Morning, August 9]

Chairman—Professor S. G. SHATTOCK, F.R.C.S.

SECTION III

GENERAL PATHOLOGY AND PATHOLOGICAL ANATOMY

DISCUSSION No. 3

GRAFTING OF NORMAL TISSUES AS DEPENDENT ON ZOOLOGICAL OR INDIVIDUAL AFFINITY: AUTOPLASTIC, ISOPLASTIC, HETEROPLASTIC

REPORT BY MONTROSE T. BURROWS, M.D.,
ANATOMICAL LABORATORY, CORNELL UNIVERSITY MEDICAL
COLLEGE, NEW YORK CITY

Tissue Culture in Vitro

THE work of biologists on lower metazoa and unicellular organisms has taught us much concerning the nature of protoplasm. In such forms the single cells or small groups of cells possess all the properties necessary for life, though some are incipient. In passing from these lower animals up the scale we see various tendencies towards specialization on the part of the cells. In the cœlenterates, for instance, one finds certain groups of cells differentiated into tactile zooids, others into defensive zooids, nematocysts, and others into nutritive and finally reproductive zooids. In all cases when the attainment of this specialization renders the cell more efficient for a definite function, other generalized life properties are usually sacrificed. Hence in the study of the mammalian body one finds numerous types of cells, each specialized for its particular function but all largely dependent upon others for their existence.

The question presents itself whether these specialized tissues might not be capable of independent existence if isolated in the proper environment. In other words, has this state of ' physiological division of labour ', which was so beautifully presented by Herbert Spencer, been actually carried to such an extent on the part of the individual cells that they are unable to revert to ancestral properties and again become capable of performing all the life functions necessary for their independent existence?

The behaviour of the body cells of the higher metazoa has been largely analysed in the light of the responses of lower organisms. The leucocytes and wandering blood cells are constantly compared to the amœba, one of the simplest protozoa. Whether or not such comparisons are legitimate must be experimentally demonstrated.

How far the differentiation and specialization have carried the cells, to what extent they have lost the properties of unicellular animals, and to what extent the tissues in their development and adult relations affect one another are questions to be answered. Certainly the most direct method of testing these problems would be some means by which it is possible to remove the cells from the animal body and cultivate them in a known and regulated environment.

Inquiries of this sort naturally led to an attempt to cultivate tissues *in vitro*. Max Schultze, in 1860, by means of a warm stage, observed and studied the movements of leucocytes in blood clots. Ranvier and others studied the movements of frog's blood corpuscles in a hanging drop of clotted lymph. Jolly, repeating Ranvier's experiments, used the blood of Triton ; he noted division of many of these blood cells. Roux had isolated the blastomeres of frogs' eggs in egg-white and salt solution, and from a study of the movements of these cells had developed his theory of ' cytotropism '.

Leo Loeb, in 1897, pointed out the value of such a method for studying other cells. Loeb placed pieces of tissue in clefts cut in blocks of agar or coagulated blood, and observed movements of the epithelium and connective tissue when these blocks were placed in test-tubes or under the skin of animals. Beebe and Ewing had seen movements of tumour cells kept apart from the animal organism in a circulating blood-stream. None of these earlier attempts at tissue culture were carried far enough, however, to show any general application.

It remained for Harrison, in 1907, in attacking a fundamental problem in biology, to fully demonstrate the value and more definite application of such a method. Two opposing views regarding the origin of the axis cylinder processes of nerves were advocated : one, that it was a direct outgrowth of the nerve cell ; another, that it formed in part from the nerve cell and in part from other cells. Harrison showed that the nerve fibre could be grown entirely separate from other cells, and that the axis cylinder process was an outgrowth of the nerve cell itself and grew by an independent activity of its protoplasm. He thus demonstrated the truth of the outgrowth theory of the nerve fibre as originally enunciated by His and further developed by Cajal.

LYMPH METHOD

Harrison introduced the isolated central nervous system as well as other tissues of young frog embryos into a drop of fluid lymph obtained from the lymph sacs of adult frogs. The drop containing the tissue was placed upon the surface of a cover-glass. The cover was inverted over a hollow slide and sealed with paraffin to prevent drying. After a few minutes the hanging drop clotted, holding the tissue firmly in a fibrin network.

Such preparations could be readily observed under the microscope. After a few hours or days at room temperature, cells wandered out from the periphery of the tissue fragment. The cells of the embryonic neural tubes sent out processes which grew progressively longer and longer. These processes, the axis cylinder processes of the nerve cells, were provided with a thickened end, the locomotive apparatus. This end was actively moving. The result of this movement was a progression of the thickened end and a drawing out and lengthening of the fibre.

Further and more definite evidence was needed to prove that such a type of growth was normal. This was found in the differentiation of embryonic epithelium as well as differentiation of the muscle cells of the myotomes.

Harrison demonstrated by these experiments that the cells of embryo frogs could survive away from the animal body and show certain definite manifestations of life, namely, movement and differentiation. If these cells could be grown it seemed likely that cells of other and higher animals could live apart from the animal organism, and with the further development of the technique many other manifestations of life might be observed. The method had been used chiefly for the study of a morphological problem, but it seemed evident that with modifications it might furnish a ready means for the study of many other problems of growth.

In the spring of 1910, through the kindness of Professor Harrison, I was allowed to take up a study of these cultures in his laboratory with the idea of more fully developing the method, so as to make it of general application for a study of cell activities. The difficulty lay, as Harrison pointed out, in the lack of a suitable medium and tissue. Lymph, which Harrison had used, could be obtained from frogs, but from these animals only in small quantities, and its consistency varied widely, at least in fibrin content, even in the different lymph sacs of the same individual. The medium which approached lymph most nearly in general consistency was blood plasma. This could be obtained from any animal, and in quantities sufficient for the preparation of a large number of comparable cultures.

PLASMA METHOD

I found by the application of the well-known laws of blood coagulation that plasma could be readily prepared. Blood was collected directly from the heart, a vein, or an artery through an oiled cannula into a tube lined with a layer of paraffin and kept cold by surrounding it with ice. The chilled liquid blood was centrifugalized by placing the tubes in large centrifuge receptacles which were filled with salt, ice, and water mixture. The supernatant plasma, when free from cells, was pipetted into other tubes and preserved in the ice-box. The plasma of frogs and chickens could be kept by such a method for days or months. Recently I have discarded the use of oil and paraffin. Clean glass-ware answers as well for the preparation of plasma from the blood of any animal, and greater cleanliness is assured.

The work was continued with tissues of chick embryos. These embryos represented a warm-blooded animal.

The growth from pieces of the tissues of chick embryos was more extensive, but essentially the same as Harrison had observed from pieces of frogs. It consisted in an extensive migration of cells into the medium about the tissue. I was able to confirm by a study of pieces of the neural tube all previous observations made by Harrison. Further studies of these and other tissues showed that cells in a culture could also perform other functions. Mitotic division, although difficult to observe in cultures of frog tissues, was most common in cells which wandered into the medium from the tissues of chick embryos. The transplanted pieces or the whole heart of these embryos often beat rhythmically for many days.

Carrel and I, during the winter of 1910 and 1911, tested the applicability of this method to the study of adult tissues. We showed that plasma could be readily prepared from all animals, and that tissue of both adult and embryonic mammals survived and showed activity in hanging-drop cultures where either plasma of the same animal or another animal of the same species was used. The animals used were chickens, rats, mice, guinea-pigs, dogs, cats, and man. Many other animals have been carefully tested by other observers. The growths included both the epithelial and connective-tissue cells. Malignant tumours were tested and their growth fully established. These observations have been fully confirmed by the work of Lambert and Hanes, Loeb and Addison, Braus, Weil, Hada, Oppel, and others.

The tissues of lower animals were cultivated with greater ease, however, than human tissues. The reaction of normal human tissues, whether embryonic, adult, carcinomatous, or sarcomatous, is practically the same. There is rapid and complete liquefaction of the plasma clot. The entire clot may go into solution within two or three hours. Small growths may be observed, but they consist only of a few cells migrated along the surface of the cover-glass. I tried in recent experiments to obtain growths by repeatedly transplanting the tissue to fresh media. This gave no result. At each transfer the medium was liquefied until the death of the tissue was in evidence. The dead tissue does not liquefy the medium, and the clots remain firm about it for several weeks.

These experiments gave no evidence of a failure of response on the part of human tissue. The cells which wandered along the cover-glass showed activity equal to similar tissues of other animals when placed in a similar environment. The failure of growth was the result of a destruction of the clot or the solid medium necessary for cell movement. The ability to digest the clot or portions of the clot is manifested by all living tissue cells so far tested. Most animal cells move abreast of this dissolution of the fibrin. One may see, however, extensive dissolution of the clot unassociated with cell movement about many fragments of tissue containing epithelium or even other tissue cells. This is especially true when the cells in the tissue fragment have been slightly injured. Whether this dissolution of human plasma is peculiar or the result of technical error is a question to be answered by further experimentation.

General Characters of Growth

The growth obtained in the cultures varies with the tissue as well as the age of the animal. A slow digestion of the clot is generally the only evidence of cellular activity noticed about fragments of adult striated muscle. The fibres have been rarely seen to move, and division of the nuclei has not been observed.

Embryonic and malignant tumour cells grow, on the other hand, with great vigour. They cause extensive dissolution of the clot ; the cells wander widely and often grow and divide.

Connective tissue in every case grows more actively than epithelium. The epithelial cells, while showing a greater ability to digest the fibrin, move more sluggishly and divide less frequently.

Connective tissue is active and grows generally as a syncytial network or isolated cells into the surrounding media. The cells are most often spindle-shaped, although irregularly shaped cells are not uncommon. All tissue cells grow quite characteristically to their kind. Epithelium from skin remains in sheets or membranes. Few cells, however, often become separated and wander away as isolated elements. During their wandering they show some amœboid-like movement. The grouping of the cells generally remains characteristic of the various sources from which they are taken. Thyroid epithelium tends generally to form tubes rather than membranes. These tubes are readily and easily distinguished from the tubular growths of kidney or other organs.

Whether embryonic nerve cells divide has been difficult to determine. The growths have been contaminated invariably with other tissue cells. These cells may migrate, however, when separated from other cells. When held firmly through attachment to tissue or fibrin they send out long processes, the axis cylinder processes. Such a growth of nerve fibres has not only been observed in cultures of embryonic tissue, but more recently by Marinesco and Minea and by Ingebrigsten in culture of the spinal ganglia of adult mammals.

The cells from the spleen, bone marrow, lymph nodes, and blood migrate most actively, but they have been seen to divide by mitosis in but one culture. Some authors claim, however, they divide by amitosis. This I have not observed with certainty except in giant cell formation.

Other functional activities and differentiation had been seen in the earlier studies only in the cells which remained in the tissue fragment.

By a more careful study last year I observed differentiation and rhythmical activity in embryonic heart-muscle cells, which had wandered out into the medium and had become separated entirely from other cells.

By the early observations of growth and differentiation of cells *in vitro* it was evident that the tissue culture was a valid method for the study of many phenomena peculiar to the life of the cell. Many attempts have been made to use this method for a study of various problems of cell metabolism, for the study of the environment influencing various cell functions, the limits of autonomy shown by these cells and the effects of various substances upon their growth.

Growth in Extraneous Media

Studies of this kind were introduced by the work of Schorer, M. R. and W. H. Lewis. Schorer showed that the nerve cells of chick embryos could be grown in agar. M. R. and W. H. Lewis confirmed this work and demonstrated growth of tissues of embryo chickens in many liquid media, such as sodium chloride, Ringer's solution, Locke's solution, and serum. It was interesting to note that the tissues grew even in widely different concentrations of pure sodium chloride, varying from two and a quarter to seventeen parts per thousand. No growth took place without sodium chloride, and it was prolonged and increased by addition of calcium and potassium. Additions of maltose or dextrose or protein decomposition products increase proliferation. Cells grew still more actively in bouillon agar, while the most luxuriant growths were obtained in plasma.

The growth in Locke's solution or more complex synthetic media was often quite extensive. A few mitotic figures were observed in the stained preparations.

Cultures

Newer technical methods aided further in this study. In the simple hanging-drop culture of Harrison, which was used chiefly, the tissue is placed in plasma or a drop of some liquid medium, which is surrounded by a given air-space. The culture chamber is carefully sealed to prevent any interchange with the outside. An active cellular metabolism must necessarily deplete the oxygen and increase the carbon dioxide, consume the food material, and increase products of catabolism. In 1912 I described a culture chamber which is so arranged as to allow an interchange of air about the tissue as well as an interchange of liquid media. The air-chamber of the culture communicates with the outside by way of a tube guarded by a moist chamber. The liquid portion of the medium is continuously changed by allowing a slow stream to flow through the culture along a cotton wick. This culture, in brief, consisted of three chambers, a supplying chamber, a culture chamber, and a receiving chamber, connected by glass tubes in the order named. The wick begins in one compartment of the supplying chamber and passes through the culture chamber into the receiving chamber. In the culture chamber it is teased apart over the surface of the cover-glass into its individual fibres. The tissue is cut into small pieces, placed among the fibres of the wick, and held there by the coagulation of a drop of plasma. The supplying chamber is built so that the medium flows drop by drop upon the upper end of the wick. The flow can be regulated as to time and quantity. This culture I call for convenience ‘ the wick culture ’, as contrasted with the former, ‘ the simple hanging-drop culture.’

STUDIES ON CELL MOVEMENT AND REPRODUCTION IN VITRO

In 1910 and 1911 Carrel and I attempted to use the tissue culture as a means for studying methods of accelerating and inhibiting the growth of tissue cells. We found that a greater growth of cells occurred in plasma which had been altered by the addition of various quantities of distilled water or tissue extracts. Carrel at a more recent time has repeated and confirmed these results, accepting our former theory that some direct effect was produced upon the cell by the added substances.

Through a more careful study of the effect of these substances and numerous controls I found, however, that wide variations in results were constantly encountered. The growth in the control cultures in many cases was equal or greater than in the cultures where altered plasma had been used. Similar increase in movement often followed the addition of other substances, such as serum, Ringer's solution, Locke's solution, or isotonic NaCl. It became clear that the method was inadequate for drawing any conclusions as to the effect of these substances without a more careful analysis of the growth and the establishment of some means of control. If these substances, when added to the plasma, produced any effect, this effect was overshadowed by the effect of some general change produced, probably the mechanical change of dilution.

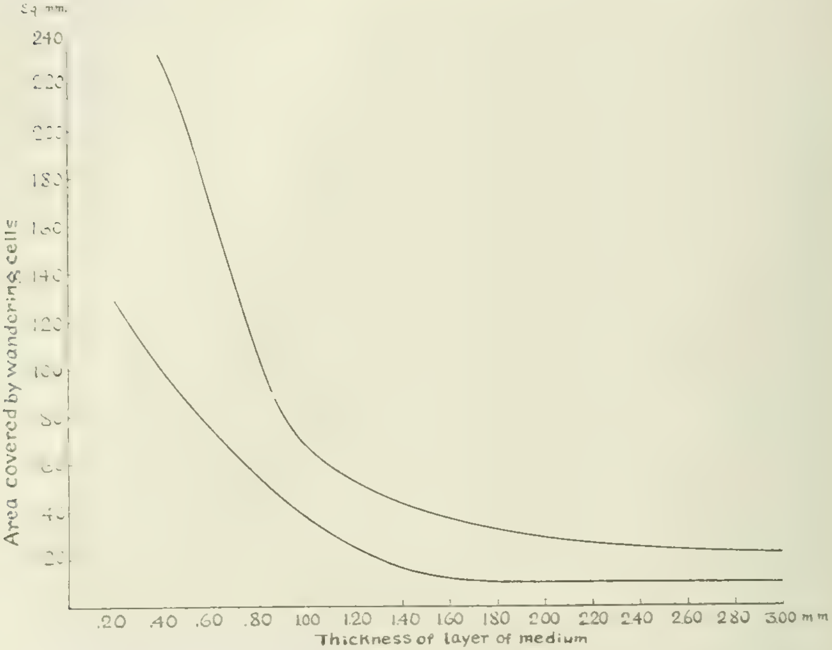
Harrison in his early analysis and later experimental studies showed that movement of cells in a culture was dependent upon contact with some solid support. Submerged in a liquid, the cells remained spherical. They spread out and migrated when placed in contact with fibrin, the surface of the cover glass, the free surface of the liquid, or any solid body.

Through a more careful analysis of this movement I have found that single connective-tissue or epithelial cells spread or flatten out when brought in contact with a surface film of the medium, but they never change their position. The extent of this spreading varies with the physical and chemical state of the medium. Cells spread slowly when placed at the surface of a fresh plasma clot. They spread, on the other hand, with great rapidity and become tensely drawn out in a liquid film resulting from a recent digestion of the clot. When the medium becomes exhausted and charged with acid waste products, they remain, of course, spherical.

Migration of these cells in a medium of uniform consistency is dependent upon the existence of other cells. One cell apparently repels the other.

The grouping and the activity of the cells, the restricting of the surface upon which they move, and a proper supply of food and oxygen must be, therefore, factors influencing the migration of cells. Oxygen from the air diffuses actively into these clots or tissue fragments no greater than 0·5 or 0·7 mm.

Through measurements I have found that the migration of cells is greater away from a large than a small mass of tissue; greater away from a cellular than a fibrous piece of tissue.[1] In the same way the surface covered by migrating cells about a given tissue depends on the thickness of the plasma layer. As an example, the greatest movement of spleen cells about a piece of tissue 1 to 1½ mm. in diameter has taken place in a layer of plasma 0·2 to 0·3 of a mm. in thickness. The surface area covered by cells about similar pieces decreases with each increase in the thickness of the plasma layer up to a certain limit. Above this limit of thickness the growth reaches a constant minimum.



Curve I. Variations in the growth of spleen cells from two sources in layers of plasma of various thicknesses.

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[1] This fact became of interest in the study of the movements of sarcoma cells. Many authors have noticed a greater movement of these cells and embryonic mesenchyme as compared with normal adult connective tissue. In most of these cases the number of cells in a given piece of sarcoma or embryonic mesenchyme is greater than in the same sized piece of adult connective tissue.

In the thicker layers of media the cells in the tissue mass early become inactive. Only a few cells wander out into the medium, and these show a low grade of activity. The cells separate and soon come beyond the influence of changes taking place about other cells. In the thinner layers, on the other hand, the cells of the original fragment remain active. A large number of cells are directed out into this thin sheet of medium, which restricts them almost to a single plane. A continuous line of cells, therefore, follows each preceding one.

In contrast to these observations which have been made on spleen cells are those on heart muscle and skin. In the case of these tissues the greatest area covered by cells about a fragment of tissue 1 mm. in diameter is attained in layers of plasma varying between 0·5 and 0·7 mm. in thickness. They suffer, apparently, from a lack of nutrition in the thinner layers.

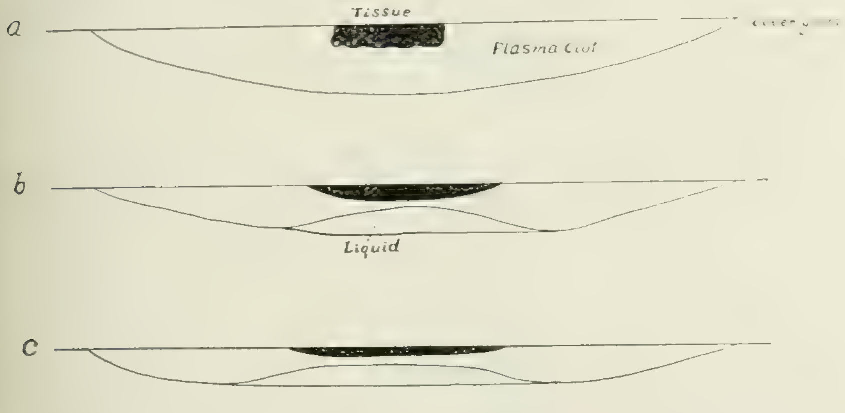


FIG. 1. Cross-section of a drop of plasma hanging from the lower surface of a cover-glass and containing a piece of skin: (*a*) Thickness of layer of plasma and position of piece of tissue at time of preparation; (*b*) section of the same culture 20 hours later; (*c*) section of the same culture 72 hours later. Magnification 7×.

The movement of both epithelial and connective-tissue cells out and away from the tissue mass is also influenced by another factor, namely, the possibility of securing a rigid support. The spleen cells do not apparently need so strong a support. They dissolve a path and move by attaching their pseudopodia to the walls of the burrow. The clot has a sufficient consistency to withstand the insignificant pull produced by movements of the spherical-shaped spleen cells.

On the other hand, in the case of epithelial and connective tissue, this pull may be considerable. Epithelial tubes or membranes form by a stretching of the epithelial layer of the fragment. This movement is associated with an extensive dissolution of the clot, which results often in the formation of a large cavity. The membrane either moves out to line the cavity or becomes stretched through it by the ability of the border cells to cling to the edge of the dissolving clot. The connective-tissue and embryonic heart-muscle cells grow in a similar manner. The migration of these cells is associated with considerable strain both on the clot and the tissue fragment.

When the fibrin is attached firmly without, lines of stress are seen to form within it between the tissue border and this point of attachment. These form before the cells move out from the tissue. When the clot is unsupported no movement results, but the clot is drawn in mass towards the tissue fragment and dissolved (Fig. 1). In hanging drops, unless the medium is of very firm consistency, migration can take place only in the direction or along the surface of the cover-glass.

The same failure of movement is seen when the tissue is unsupported.

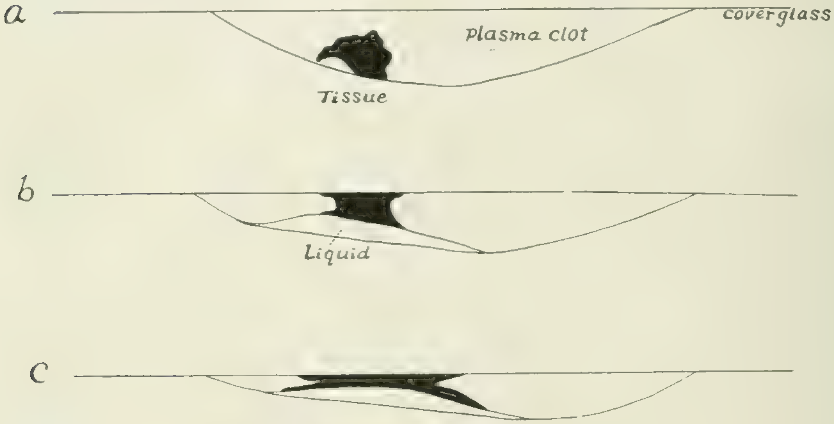


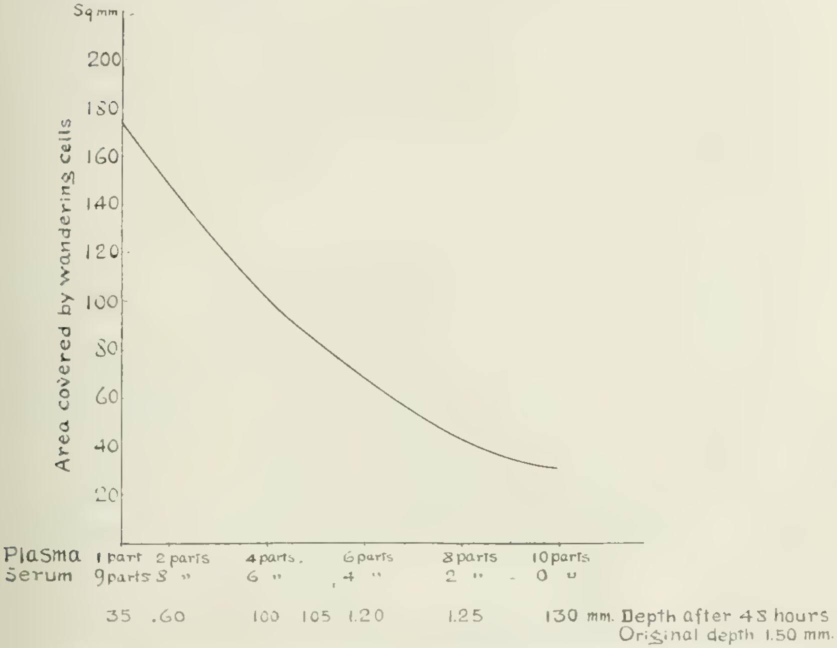
FIG. 2. Cross-section of a drop of plasma hanging from the lower surface of the cover-glass and containing a piece of skin: (*a*) Thickness of the layer of plasma and position of the tissue at time of preparation; (*b*) cross-section of the same culture 20 hours later; (*c*) cross-section of the same culture 72 hours later. Magnification 7×.

Cellular connective-tissue fragments of a loose texture are drawn out into thin sheets. Very few cells wander from their periphery. The cells migrate away from the firmer fragments. When the fragment is entirely unsupported it is carried along in mass, no cells appearing from it unless it meets resistance (Fig. 2). I have frequently seen pieces of skin, when broken loose on all sides except one, travel long distances across the culture. The wide area of dissolution to the sides and behind them appears like that of a boat ploughing its way through the water.

The dissolution of the clot about an epithelial layer is often so extensive as to inhibit any growth of the neighbouring connective tissue. In a piece of skin rich in connective-tissue cells the liquefaction of the fibrin may be in turn associated with a failure of growth of a membrane of epithelial cells. In thick layers of medium this liquefaction often widely separates the connective cells from the epithelial membrane.

In the light of these experiments it became possible to control more completely the result obtained from the addition of various substances to the medium, and to determine how dilution may affect movement, and what effect the various diluents may have on the growth of the tissue.

Dilution may alter the growth in at least two ways: first, by allowing a greater contraction of the clot, so surrounding the tissue by a thinner layer of solid medium which promotes or inhibits growth, as the case may be. Second, in those clots which fail to contract completely the decrease in the density of the clot may allow a greater movement of connective-tissue or epithelial cells along the cover-glass or spleen cells through the midst of the clot.



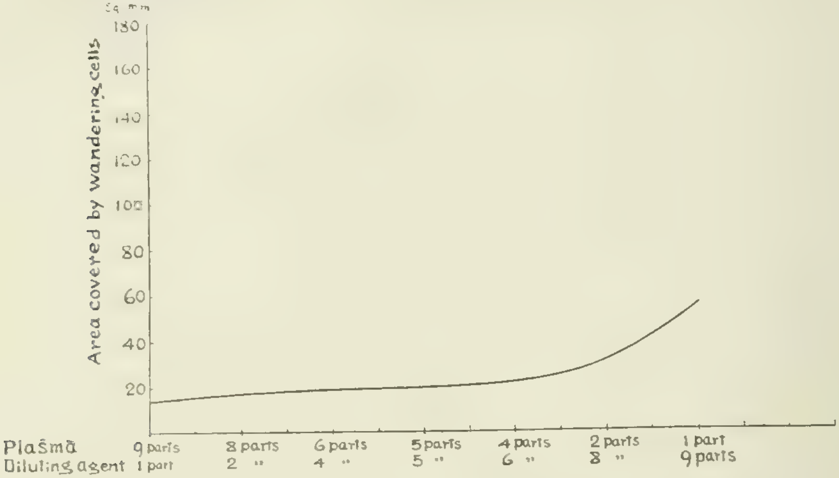
CURVE 2. Variations in the growth of spleen cells in equally thick hanging-drops of various diluted plasmas.

Clots have undergone the most complete contraction after the use of serum or tissue extracts. The relation of the extent of this contraction to the increase in movement of cells subsequent to each increase in the quantity of diluent added to the plasma is seen by a comparison of Curves 1 and 2.

The effect of a decrease in density is shown by Curve 3. In this case similar pieces of spleen were placed in thick layers of pure plasma, and plasma altered by the addition of some diluting agent. To prevent contraction and the diffusion of oxygen, the layers were superimposed between glass plates.

Aside from these mechanical changes the addition of distilled water or salt solutions is harmful. Isotonic NaCl accelerates often movement and rhythmical activity in heart muscle, but the decrease in other constituents of the plasma, as the result of its addition, inhibits generally any extensive growth or division of the cells. Serum and tissue extracts, on the other hand, when added to the plasma, produce changes often of advantage for movement, and allow, as a rule, a subsequent growth of the cell equal to that observed in pure plasma.

Direct application of many facts so derived to wound healing was facilitated by a method introduced by Ruth. Ruth found that if holes were cut in pieces of frog's skin and these pieces placed in a layer of fibrin, the wound would heal. This healing took place in every case from the epithelium. The cells wandered out and bridged the gap. Later, the fibrous edges, previously remaining inactive, moved together.



CURVE 3. Variations in growth of spleen cells in different diluted plasmas.

CELL REPRODUCTION

From the work of Peter, Nussbaum, Barfurth, Leo Loeb, and others it is well known that cellular migration is a primary phenomenon in wound healing. From the cells which wander out, bridge, and fill the gap, new cells are formed.

This stretching and flattening of the cell is apparently a necessary factor for growth and division in a culture. Only those cells grow and divide which are flattened out so they present the largest possible surface to the surrounding medium and one diameter reduced to a minimum.

Not only the spreading but likewise the growth of the cell depends further upon a complete change of the clot. Cells in contact with a very thin layer of unchanged fibrin have never been seen to grow or divide. Dividing cells are spread either over the surface of the cover-glass or a thin network of hyaline fibrin fibrils within an otherwise liquefied area of the hanging drop.

It has been interesting to note that the growth of cartilage cells depends on a like spreading of the transplanted fragment. In the case of this tissue the cells take apparently little active part in the spreading; the matrix clings to the dissolving fibrin. Cubical pieces of newly formed cartilage may spread out into broad thin sheets. The cells within thus becoming separated and flattened show often evidence of growth.

OTHER FUNCTIONS IN TISSUE CELLS

Using embryonic heart muscle for this study it has been found that even in richly nutritious surroundings slight changes in the attachment of the cell to the solid substances about it may derange completely its function. The elongated cylindrical-shaped heart-muscle cells held by the attachment of one end to the tissue and the other to the fibrin or stretched between the cover-glass and a strand of fibrin do not grow and divide, but generally differentiate and beat rhythmically.

FURTHER STUDIES ON CELL LIFE IN VITRO

To maintain a given type of functional activity in a given cell a carefully arranged technique is necessary. Not only must the cells move so as to obtain a support suitable for a particular function, but a proper relation must exist between the size of the tissue fragment and the quantity of medium. The dissolution of the thinnest layers of plasma is not sufficient about small pieces of tissue to allow the cells to grow. When large pieces of tissue are placed in a thick layer of plasma, only a part of the clot is dissolved. Heart-muscle cells wandering into these clots show neither division nor rhythm. When coming to rest they become inactive. In this state, however, they may remain for a very long time provided sufficient plasma surrounds them.

Last May I placed 1 mm. cubes of heart muscle, skin, and spleen in layers of plasma ranging between 1½ and 2½ mm. in thickness. The medium and the tissue were hung from a cover-glass over a small hollow chamber, carefully sealed with vaseline and paraffin, and incubated. During the first two weeks cells wandered out into the medium. At the end of this time all movement ceased, but the cells in the clot did not disintegrate. Six months later the cells which were flattened against the cover-glass and the lower surface of the clot were dead. Cells in the midst of the clot of many of the cultures were still the same as at the end of their wandering period. Not even the arrangement or the number of fat droplets within many of them had changed during the last few months. Upon transplanting I found that the centre of the original tissue mass was dead. The peripheral cells and those within the clot were living and grew actively in the fresh plasma and oxygen supply (Fig. 3). Braus noted that some of his cultures of frog tissue were alive three months after he had prepared them

Ljunggren kept human skin alive for three months by immersing it in sterile ascitic fluid. Many of the cells which appeared dead grew when they were grafted upon a denuded surface. Jaques Loeb has noted that unfertilized eggs do not disintegrate when kept at the bottom of a test-tube of liquid. When brought into contact with oxygen they die unless fertilized. The same is true in part for these cultures. When fresh air is added to the culture chamber, in a way so as not to cause drying or concentration of the medium, disintegration may or may not take place. If the clot be small and nearly exhausted the cells are often killed at once. Change of air has no effect when a large area of clot surrounds the cells.

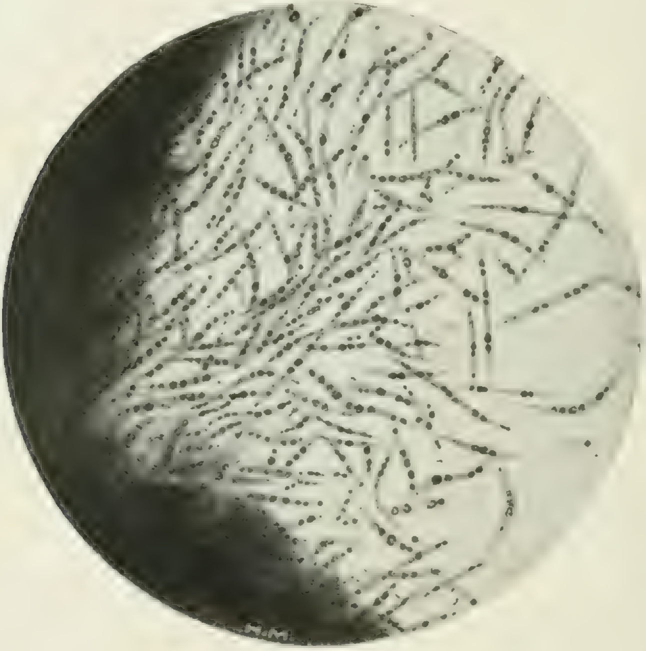


FIG. 3. Growth of connective-tissue cells from a piece of skin kept six months in a hanging-drop culture at 39·4° C.

Carrel and I, in 1910, transplanted the original piece of tissue from a plasmatic culture, which had grown actively for some time, to new plasma. We found that the border cells of this mass of tissue, which had remained more or less inactive at the first transplant, now wandered out into the media. Secondary and tertiary cultures grew actively. The scattered and newly grown cells were placed in fresh medium, but little evidence of activity was noticed. They may show movements leading to their further separation, but they soon become inactive and loaded with fat droplets. Carrel and Lambert, working independently, tested more completely this method of transplanting the original tissue to fresh media. Carrel has a piece of the heart muscle living after 120 days. After many replants these pieces of tissue have shown rhythmical beating. Cells grew out from the tissue at each transplant. Lambert found cells dividing in a culture which had grown from a piece of tissue kept three months by repeatedly transplanting it.

The growth that takes place in such cultures varies with the technique and the tissue used. An actively growing tissue placed at each transplant in a thin layer of medium becomes entirely dissipated after the second or third removal to fresh media. It may take months and many transplants for all the cells to wander away, on the other hand, from a piece of tissue containing less active cells, especially if they have been kept in thick layers of medium.

Carrel, in other experiments, has not removed the tissue from the culture, but has washed it with Ringer's or other solutions and added new medium. In these cases he states that a considerable increase in tissue was noted, especially if tissue extracts have been added to the medium. He gave no accurate measurements for this assumption, and so it remained difficult to determine whether the increase was actual or only apparent, due to a stretching of the tissue mass.

By the use of the 'large wick culture' I have found that cells may be maintained in a continuous state of function for a time. Pieces of tissue 1 mm. in diameter may become completely dissipated in this culture. The movement of cells is greatest against the stream, and in this direction they may move slowly for a very long period of time. Division is uncommon. It has been seen most frequently in a few cultures where the stream of serum was stopped for a time. Heart-muscle cells beat actively, on the other hand. In the 'simple hanging-drop culture' the rhythm becomes irregular after a few days. I have kept heart-muscle cells beating with a regular rhythm in the 'wick cultures' for as long as one, two, and even four weeks, where freshly-prepared blood serum was used as the medium.

At the end of this period the cells become scattered. The clot is largely dissolved. The cells are found floating in the medium or spread out over the surface of the wick fibres or the surface of the cover-glass, where they are inactive. In some of the cultures, the cells during their migration accumulate in considerable numbers within a tangled area of the wick. If such masses of cells be removed to fresh blood plasma where a new fibrin support is given them, they show movement and separate again from each other.

Fat in Tissue Cells

Fat droplets in the cytoplasm of cells growing in a culture are so commonly seen that they have been considered to be more or less normal morphological characters. They occur in all cells that have been cultivated, the nerve fibre being the sole exception. I have found, however, by recent experiments that if a tissue be placed in a suitable environment to allow active growth and reproduction, the cells are almost or entirely

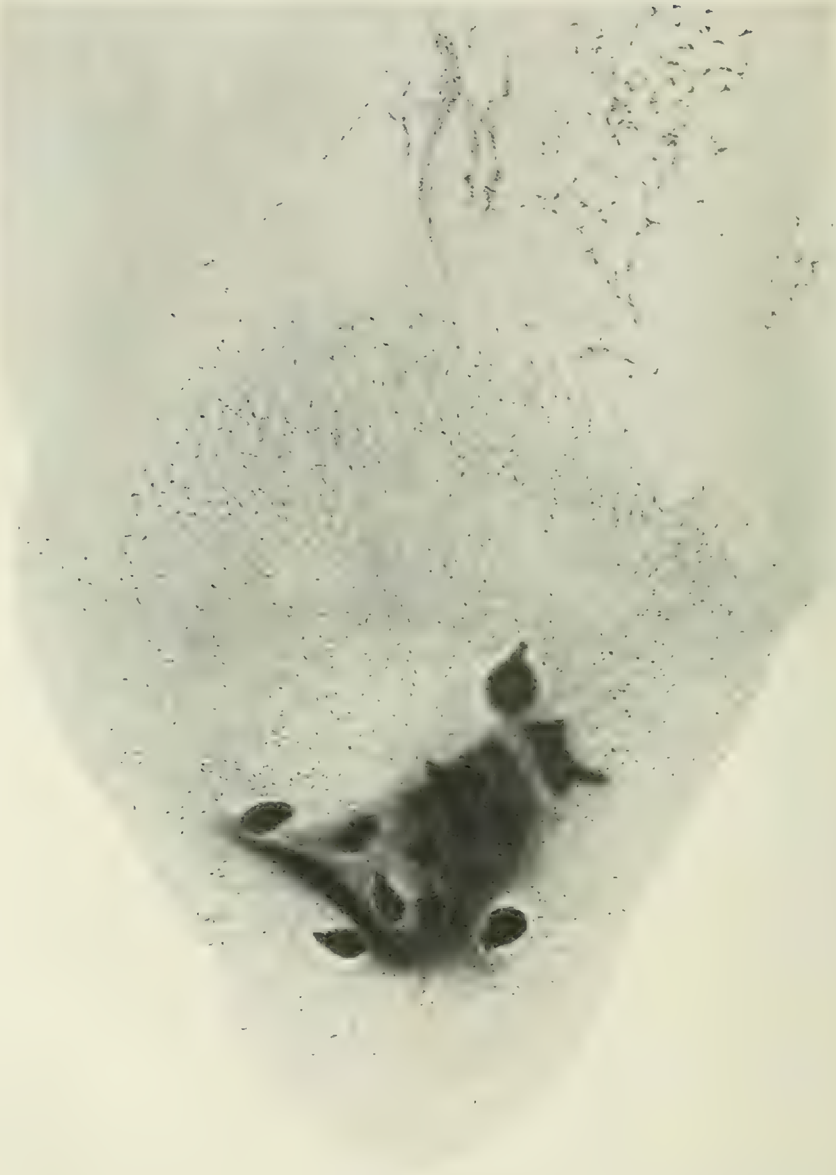


FIG. 4. Growth about a piece of skin placed at the thin edge of a drop of plasma. Fat is shown by heavy black dots in the cells. It occurs only in those cells in contact with the undissolved plasma.

fat-free. Even the cells which originally wandered out into this clot and synthesized fat may again lose it.

The quantity of fat varies in the different types of cells used. The cells from heart muscle, liver, and spleen invariably contain fat even in the best environments, while epithelium and connective-tissue cells of skin may often be fat free. The dissolution of the fibrin clot was much more rapid about the pieces of skin than about other tissues, and it was only associated with this complete dissolution of the clot that the cells lost or never showed fat (Fig. 4).

A more careful study has shown, therefore, that the fat content of cells of a culture varies similarly to cells in the body ; i. e. either from a change of cell activity or from variations in the amount of fat supplied to the cell. M. R. and W. H. Lewis noted a decided decrease in the fat content of cells growing in fat-free media, such as simple salt solutions. The fat is greater in cells growing in plasma obtained from the blood of animals suffering from anæmia or animals fed upon fatty foods previous to bleeding. There is, likewise, a sharp decrease in the fat content of cells growing in plasma diluted with salt solutions as compared with the same plasma diluted with serum or used whole.

Many disturbing factors lead to an increase in the synthesis of fat within the cells of a tissue culture, such as bacterial infections, increased concentrations due to slight drying or additions of toxic agents, or decrease of oxygen, &c.

Further Studies on Cell Division

On account of the ease of observation and the type of cell used the tissue culture became important for studying factors concerned in the division of cells. From the work of Lambert it became evident that many of the abnormal mitoses of tumours may be studied by this method.

Last year, as I pointed out, the cells flattened out over the surface of the cover-glass may show amœboid-like movements during their division. Many authors using the kinematograph have pictured such processes in the daughter cells subsequent to their formation. Jolly had seen them in his studies on division of blood cells of Triton.

I found by a more careful study that amœboid-like movement of the cell occurred not only subsequent to cleavage but often with it and at the poles of the mother cell, a fact of importance in elucidating the point of primary movement causing cell cleavage.

Bütschli following Quinke showed that cell division finds an analogy in the surface-tension phenomenon in other liquid substances. Bütschli assumed that the cleavage furrow was an area of the greatest surface tension. Whether this area of increased surface tension was the result of an actual increase at this point or the disproportion resulting from a decrease in surface tension at the poles of the cell was a question to be answered.

A further study of these processes became of interest in demonstrating the relation of movements in dividing cells to changes taking place in and about the nuclear area. Such processes have been observed in the early as well as the later stages of division. They occur always along that part of the wall nearest the nucleus. To compensate for their formation a near part of the wall is often constricted. This part slowly bulges again, a permanent constriction resulting at some point most distant from the nucleus. These facts were well illustrated by the study of cells having eccentrically placed nuclei. In the case of these cells one line of chromosomes comes near one border of the cell. Upon this approach amœboid-like processes form along the wall. At first they form slowly, the rate and the extent of their formation increasing with the approach of the nucleus to the wall. Contact of the nuclear half with any part of the wall is followed invariably by an explosive bulging at this point.

These experiments indicate that cell cleavage is, in part at least, the result of a bulging of the cell at the poles, and, further, the bulging increases inversely with the distance of the nucleus from the cell wall, a fact of importance for the theoretical interpretation of the mechanism of cell division.

Automaticity of the Heart-muscle Cell

The observation of normal rhythmical activity in isolated heart-muscle cells presented a solution of the long-disputed question in physiology concerning the nature of the heart-beat. Haller, in 1757, taught that the heart was an organ independent of the nervous system ; Remark, in 1848, through the discovery of nerves in the frog's heart, held that the heart rhythm was dependent upon the nervous system. The view was strengthened by the discovery of ganglion cells within the heart muscle. The difficulty that confronted Engelmann, Gaskell, and others in establishing a direct proof of the myogenic theory through a study of beating pieces of heart muscle lay in the difficulty to rule out the existence of functioning ganglion cells. Similar objections were raised by the neurogenic school against the observations of rhythm in embryonic chicks' hearts previous to the ingrowth of nerves from the central nervous system. My observations of persistent rhythm of single isolated heart-muscle cells add further evidence in favour of the myogenic theory. They show that heart-muscle cells removed from hearts which are well supplied with nerves can beat with a rhythm similar to that seen in this heart when in the animal body.

Growth of a Tissue in Plasma of the Same Animal and in Plasma of Another Animal of the Same Species

The growth of a given tissue in the plasma of the same animal may not be different from that in plasma of another animal of the same species.

In other cases, as I have shown in curves for the growth of the spleen (Curve 2), the variation may be considerable. Tissues from different sources may react differently to the same plasma. The tissues of an unhealthy animal frequently grow more actively in a plasma obtained from a healthy young animal than in their own plasma. The plasma of healthy young animals is a better medium for tissue than that of old animals or vice versa. A greater difference exists between plasmas taken from animals recently fed and those which have not eaten for several hours. No careful study has as yet been made upon the cause of many of these variations, yet they may furnish a possible means of furthering our knowledge as to the relation of nutrition to growth.

Growth of Tissues in the Plasma of an Animal of a Different Species

The differences seen by a comparison of the growth in the plasma of an animal of a different species as compared with those seen in plasma of the same animal or another animal of the same species are striking. Lambert and Hanes, Carrel and I, and others have noted that the tissue of several animals will grow for a considerable time in the plasma of other and often widely different species. I noted in my early experiments an active growth of the tissues of salamanders in the lymph of frogs. Lambert and Hanes continued these studies. They find that the plasma of rats and mice can be interchanged as a culture medium for tumours and normal tissues of these animals. The greatest activity always occurs, however, in the auto- or homogenic media. Rat tumours grow very well in guinea-pig plasma. By repeatedly transplanting the tissue, Lambert and Hanes have found that growth continues actively for as long as thirty days. Rat cells grow slowly in rabbit plasma, but they are not rapidly killed. Tumour cells of rats grow when inoculated into animals after remaining for twelve days in rabbit plasma. These tumour cells show movement in dog plasma for two days, after which they disintegrate. Rat tumour does not grow in goat plasma, and the cells are dead after twenty-four hours' sojourn in this environment.

Guinea-pig and mouse plasma form, apparently, a suitable medium for the survival of the cells of rats over long periods. Dog and goat plasma, on the other hand, contain substances toxic for the rat cells. Ingebrigsten has found that guinea-pig leucocytes move more actively in heated goat serum than in the unheated. These experiments suggest that the inhibiting substance is of the nature of a thermo-labile cytotoxin.

Although under normal conditions mouse and guinea-pig plasma are suitable for the survival of the cells of rat tumour or tissues, it becomes unsuitable or toxic when the animals from which it is obtained have been previously injected with an emulsion of any rat tissue. Lambert and Hanes have tested the plasma from guinea-pigs and found it suitable for the growth of rat tissue. These pigs were then injected with blood or emulsions of various cells of the rat. After a period of several days plasma was taken from the animals and used as a culture medium. This plasma inhibited growth and killed the cells of the rat.

Carrel and Ingebrigsten have been able to produce hæmolysins in a tissue culture. They placed washed red cells of the goat in cultures of guinea-pig bone marrow and lymph glands in guinea-pig plasma. After a few days the wandering cells from these tissues of the guinea-pig engulfed the red cells of the goat. The fluid extracted from the cultures was found to be actively hæmolytic for goat corpuscles. Fluid from control cultures which grew an equal time without the red cells of goats did not hæmolyse goat corpuscles.

The plasma of some animals allows primarily a rapid growth of certain foreign tissues, but does not preserve the life of the cells over any long period of time. Lambert and Hanes have found that rat tissues grow actively when first placed in the plasma of pigeons. At the end of the growth in the first culture they removed the tissues to fresh pigeon plasma and found that it did not grow. When, on the other hand, they removed it from the pigeon plasma to rat plasma, it grew. Taking it from the rat plasma and placing it again in pigeon plasma it grew again. By such zigzag transplantations the cells may be said to survive for long periods of time. Mitosis in these rat cells is as common or more frequent during the sojourn in pigeon plasma than in rat plasma.

Many interesting changes in the form and arrangement of the cells in the different plasmas are seen. These differences are due in part to the new physical as well as chemical conditions imposed on the cells. Lambert and Hanes found that large numbers of giant cells formed in cultures of rat spleen when human plasma was used as a medium. This plasma underwent a rapid liquefaction in the presence of rat tissue, as it also did in the presence of human tissues. They attributed the formation of giant cells to the fact that with this liquefaction of the clot the cells were brought against a foreign body, the cover-glass. Lambert, by a study of giant cell-formation in homogenic media, tried to show that these cells were similar to foreign body giant cells in the body. He repeated the experiments of Marchand, using lycopodium spores as a stimulating agent. As Marchand had observed, spleen cells congregate in large numbers about the spores. These cells often flow together, coalesce, engulf the spores, and form immense masses of protoplasm containing many nuclei.

Multinuclear cells in tissue culture may form from a large number of different kinds of tissues. Most of these do not form by a coalescence of cells, but through amitotic division of the nucleus without protoplasmic cleavage. Lambert has observed some multinuclear cells forming by a mitotic division of the nucleus. This is not infrequent during the late history of the culture. Contact with foreign bodies is often associated with amitotic nuclear division without cytoplasmic cleavage, though similar cells may be seen in deep layers of plasma.

CONCLUSIONS

In the foregoing discussion I have tried to present the method of tissue cultivation as it is now known, and to show how cell movement and other cell activities are in part reactions to physical and chemical environments. These studies may be of importance in establishing methods for the more careful solution of cellular activities. Through studies of growth in plasma we may also arrive at a more logical and systematic method for the treatment of wounds and the grafting of tissues. I wish to thank the International Congress of Medicine and Professor Dudgeon for giving me the opportunity to present this subject to you to-day.

2=1

On the Parallelism between Increase in Permeability and abnormal Development of Fish Eggs.

(Contribution from the U.S. Bureau of Fisheries, Woods Hole, Mass., and the Anatomical Department of Cornell University Medical College, New York City, Prof. C. R. Stockard, Direktor.

With 4 figures in text.

(Eingegangen am 22. Januar 1914.)

The morphology of abnormal embryos of the marine teleost fish, *Fundulus heteroglitus*, has been studied by STOCKARD[1]. My work has been merely an attempt to find the cause of the abnormalities in fish eggs, and several premature papers have already been published[2].

The present paper deals mainly with *Fundulus*, but the conclusions are supported by work on the eggs of invertebrates and other vertebrates.

The abnormal development of the eggs of *Fundulus* may produce an indefinite number of different forms. Some of the most striking abnormalities of the organs seen in the living embryo approach the following types: one eye may be absent (monophthalmia asymmetrica, fig. 1). The two eyes may be represented by one median eye (cyclopia,

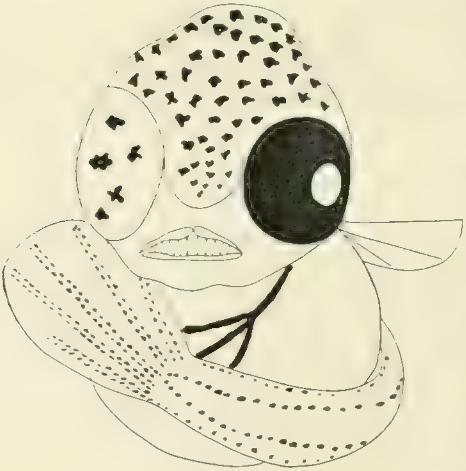


Fig. 1.

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[1] STOCKARD, Jour. Exper. Zool., III, p. 99, 1906, and IV, p. 165, 1907, and VI, p. 285, 1909; Amer. Jour. Anat., X, p. 369, 1910.

[2] McCLENDON, Soc. Exp. Biol. and Med., VII, p. 38, 1910; Amer. Jour. Physiol., XXIX, p. 289, 1912; ibid. XXXI, p. 131, 1912.

fig. 2), various cavities, such as the segmentation cavity, pericardium and ear vesicles may be distended (figs. 2 and 3). The heart may be rudimentary (fig. 3). The blood vessels may be rudimentary or apparently absent (fig. 3). The circulation of the blood may be absent. The heart may not beat. There are other abnormalities which I have omitted, but a large percentage of abnormal *Fundulus* embryos show one or more of the above abnormalities or transitions between those types and the normal.

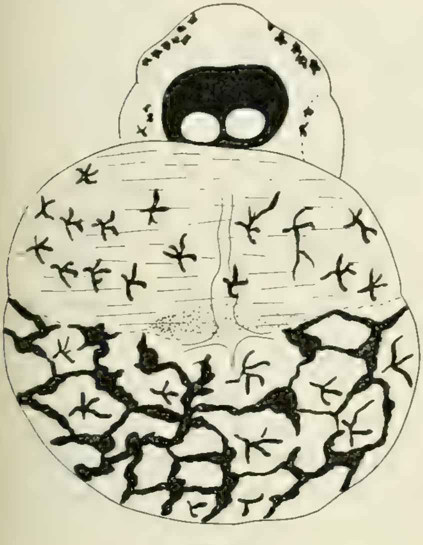

Fig. 2.

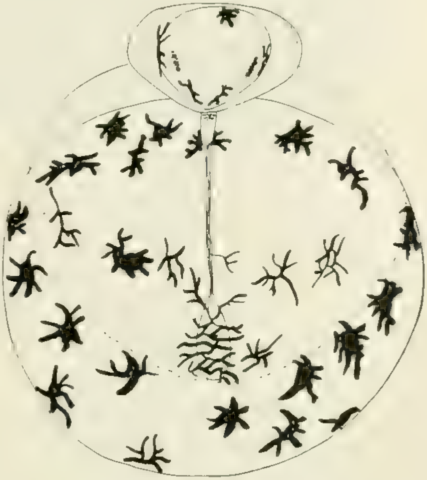

Fig. 3.

Last season I produced all of the above types of abnormalities in *Fundulus* embryos by treating them in early segmentation stages with pure solutions of any one of the following substances: Nicotine or the chlorides or nitrates of sodium, potassium, lithium, calcium or magnesium. Sea water solutions of these substances produced the same results. I thought this similarity of action of such widely different substances might be due to their action in increasing the permeability of the eggs to an abnormal degree.

It was found that the fertilized eggs of the smelt or of *Fundulus heteroclitus*, in distilled water, do not give up salts to the water. This is also true of these eggs when placed in salt solutions in which the kations are »balanced«. Thus, in an artificial sea water made of the nitrates of sodium, potassium, magnesium and calcium, no chlorides diffuse out of the eggs.

When the eggs were placed in pure solutions of nicotine or of salts of sodium, potassium, calcium, or magnesium, salts diffused out of the eggs and were detected. The detection of some salts or ions was attended with difficulties, as given in the section on technique, and it is obvious that if sodium chloride, for instance, diffused out of the eggs into a pure solution of sodium chloride, the most delicate quantitative methods might not suffice for its detection.

Absolutely trustworthy results were obtained on the diffusion of chlorine and magnesium ions, especially in the case of *Fundulus* eggs, the experiments with smelt eggs being less numerous. The results on the diffusion of sodium, potassium and calcium are based on isolated experiments (i. e., not repeated). The following table demonstrates the increase in permeability of *Fundulus* eggs.

Pure solution in which eggs were placed	Ions which diffused out of eggs and were detected
Nicotine	Cl, Mg, Ca, Na, K
NaCl	Mg, Ca, K
$NaNO_3$	Cl, Mg, Ca, K
KCl	Mg, Ca, Na
KNO_3	Cl, Mg, Ca, Na
$Mg(NO_3)_2$	Cl, Na, K
$Ca(NO_3)_2$	Cl, Na, K

Though a number of different concentrations of these solutions were found to be effective, they will not all be given, as this phase of the subject was not completely worked out. Let it suffice for the present to say that the following concentrations in distilled water were effective on *Fundulus* eggs:

KCl or KNO_3	.2 molecular
NaCl or $NaNO_3$	.3 »
$Mg(NO_3)_2$	.3 »
$Ca(NO_3)_2$	.15 »
Nicotine	.002 »

Each of these solutions, when applied in early segmentation stages, caused all of the types of monstrosities listed above. All concentrations of these solutions which caused these monstrosities, increased the permeability of the eggs. Furthermore, the permeability of the eggs was slightly increased by certain solutions too weak or acting for too short a time to produce monstrosities. Therefore, the increase in permeability is the cause or is associated with the cause of the monstrosities and is not merely an effect, i. e., one of the characteristics of monstrous embryos.

The increase in permeability probably occurs in the hypothetical "plasma membrane", as the shell or chorion is permeable to salts and water, though not freely permeable to sugar.

Possibly the permeability of certain cells or parts of the egg is increased allowing abnormal diffusion of some substance such as oxygen, CO_2, sugar or amino acid. Or the free diffusion of ions may abolish the electric polarization shown by HYDE[1] and increase the surface tension.

Material and Methods.

There are many sources of error in the electrolytic, plasmolytic and microchemical methods for determining permeability and I decided to use chemical methods. Failing to find chemical methods that could be applied to the eggs of invertebrates I chose the eggs of the smelt and of *Fundulus heteroclitus*.

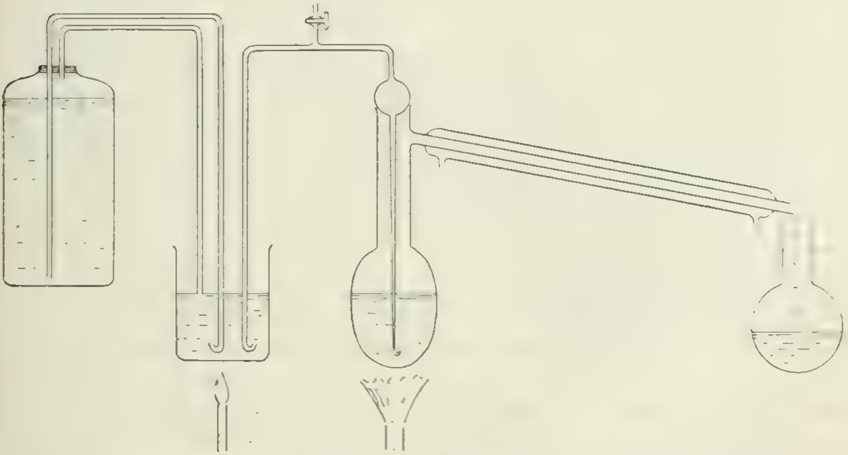

Fig. 4. Quartz still.

Clear spring water, distilled in a tin lined still, is placed in the stock bottle to the left, and from this it syphons over into the beaker. A small flame under the beaker boils out CO_2 and some other gaseous impurities. The water syphons over from the beaker into the kjeldahl flask. A bulb is blown on this syphon to close the mouth of the flask. A hole is blown in the neck of the kjeldahl flask and reamed out while soft to admit the end of the quartz condensing tube. Only water that condenses in the quartz tube can drip into the quartz flask at the right.

The eggs of the smelt will develop normally in redistilled water and in sea water diluted to about 4 times its original volume.

The eggs of *Fundulus* will develop in redistilled water, sea water or even in sea water concentrated to $^1/_2$ its original volume.

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[1] Am. Jour. Physiol., XII, p. 241, 1904.

The eggs of both species appear to be impermeable to water and to salts. When placed in redistilled water salts do not diffuse out of them unless the permeability has been artificially increased. It might be supposed that the salts were held in chemical combination or in indiffusible form in the eggs, and this is probably true of some phosphates, for instance, but omitting phosphates and carbonates of alkaline earth metals, the egg of *Fundulus heteroclitus* contains as high a percent of soluble ash (3,18) as the local sea water (2,84—3,29). Furthermore Höber has brought forward much evidence to show that at least a part of the salts in living cells is in a freely diffusible form and therefore their retention by the cell may indicate impermeability.

Ordinary distilled water was found to be slightly toxic to the eggs, and therefore all of the water used in the experiments was redistilled in quartz in the still shown in fig. 4.

The general method is best explained by describing one experiment. Fifty cc. of smelt eggs taken as soon as possible after fertilization were washed in repeated changes of redistilled water and the time when silver nitrate no longer gave an opalescence to the washings noted. The washing was then continued about 2 hours longer. The mass of eggs was then divided into 2 equal parts (a and b) and treated separately as follows:

a (control)	b
25 cc. eggs placed in 500 cc redistilled water in quartz vessels for 30 minutes.	25 cc. eggs placed in 500 cc. redistilled water plus .6 gram $NaNO_3$ in quartz vessels for 30 minutes.
400 cc. water poured from eggs and evaporated in quartz to 50 cc. and .6 gram $NaNO_3$ added.	400 cc. solution poured from eggs and evaporated in quartz to 50 cc.
1 cc. N/10 $AgNO_3$ added, solution remains clear.	1 cc N/10 $AgNO_3$ added, forming dense white cloud, due to chlorides that had diffused out of eggs.
Eggs kept in redistilled water and development watched.	Eggs kept in the remaining 100 cc $NaNO_3$ solution and at intervals of time some are transferred to redistilled water and development watched.

A separate experiment was made for the detection of each ion.

The quantities of *Fundulus* eggs were necessarily less (usually about 10 cc). This disadvantage was partly compensated by exposing them for a longer time, but owing to the disadvantages of exposing them for a very much longer time, the operation was done on a smaller scale, each solution being concentrated in a platinum crucible to 2 cc. or less before analysis.

In the event that some eggs died or bacteria attacked their surfaces or membranes, the experiment was thrown out. Even if bacterial action was too slight to give opalescence to the solution, haptogen membranes or coagula were noted on boiling down the solution and it was thrown out. The presence of a trace of reducing substance was not considered sufficient to vitiate the results, since such titrations as with silver nitrate were done quickly, and, even though allowed to stand a reduction would be detected by a change of color. Furthermore. all precipitates were examined under the microscope and the form of the crystals studied.

Most reliance was placed on precipitation methods, but these will not be discussed here as it is hoped to work them out to a fine point quantitatively in the future.

In addition to these precipitation methods a prism spectroscope was used. A grating spectroscope was found too dim for such small quantities of substance. In observing the spectrum of magnesium, high potential electric sparks were allowed to pass from the surface of the solution to a platinum electrode. In locking for other elements a platinum loop with or without a bit of asbestos wool was dipped in the concentrate solution and held in the flame observed with the spectroscope. The asbestos wool, after being thoroughly cleaned could be used only once. The platinum loop was dipped into pure hydrochloric acid and heated to a white heat and this process repeated about seven times, or until it gave no sodium or other lines to the flame. Traces of sodium were always present in the air. Although the Bunsen burner was washed in many changes of distilled water and dried, and the gas was filtered through packed cotton wool, minute flashes of sodium light appeared in the flame and were caused by sodium in the air drawn into the Bunsen burner.

The chemical reagents were the purest that could be obtained and were tested immediately before being used. Minute traces of impurities could not lead to erroneous conclusions owing to the character of the control, but they might, and in some cases did lead to the negative results, and lead me to discontinue experiments begun in 1911.

Conclusions.

Distilled or sea water solutions of nicotine or the salts of Li, Na, K, Ca or Mg all produce the same abnormalities in the development of certain fish eggs placed in them in early segmentation stages.

These substances increase the permeability of the eggs to salts or their ions (the eggs being impermeable to salts in pure water or "balanced" salt solutions).

Since the increase in permeability occurs before the abnormalities appear it is probable that the abnormal permeability is the cause of the structural abnormalities.

Zusammenfassung.

Werden bestimmte Fischeier in frühen Teilungsstadien in Lösungen von Nikotin oder den Salzen von Li, Na, K, Ca oder Mg in destilliertes oder Seewasser eingesetzt, so werden in allen Fällen dieselben Mißbildungen in ihrer Entwicklung hervorgebracht.

Während die Eier in reinem Wasser oder in ausgeglichenen (balanced) Salzlösungen für Salze undurchlässig sind, wird eine Durchlässigkeit der Eier für Salze oder deren Ionen durch obige Stoffe herbeigeführt.

Da diese Permeabilitätsänderung eintritt, bevor die Mißbildungen erfolgen, so ist jene abnorme Durchlässigkeit wahrscheinlich die Ursache der Mißbildungen.

The Laws of Surface Tension and their Applicability to Living Cells and Cell Division.

By

J. F. McClendon.

Contribution from the Embryological Laboratory of the Cornell University Medical College.

With 10 figures in text.

Eingegangen am 21. April 1913.

Quincke long ago interpreted certain movements of drops of fluid as due to surface tension changes and suggested that this might also apply to living cells. Bütschli extended this application to cell division. However, since Biologists are not all of the same opinion on this subject, I have ventured to review it from the standpoint of dynamics, based on the molecular hypothesis. Some of the principles for which dynamical proofs are given in this paper were first established by thermodynamical proofs. However, it seems simpler to omit thermodynamics wherever unnecessary. This paper claims no originality, but references to original sources are omitted for the sake of brevity, the author taking in such cases the whole responsibility for the correctness of the deductions. Since T. B. Robertson has opposite views from those of Quincke and Bütschli, it seems desirable to consider especially Robertson's latest paper[1] and all page numbers refer to his article.

Within a liquid, the molecules are attracted by one another, but the force of this attraction decreases rapidly with the distance. According to Quincke, molecular attraction is not appreciable at a distance greater than .05 μ. If a sphere be described about a molecule, with a radius equal to the range of molecular attraction, we may call it the sphere of molecular attraction.

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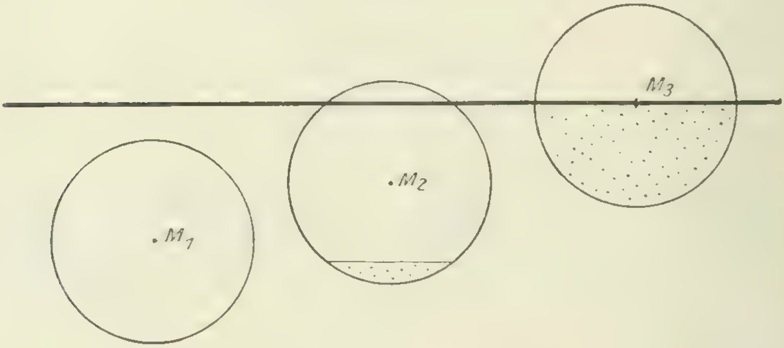
[1] Arch. f. Entw.-Mech. Bd. 35. 1913. S. 692.

In Fig. 1 the upper surface of a liquid is represented by the horizontal line and 3 molecules, M_1, M_2, M_3, by dots around each of which is a circle representing the sphere of molecular attraction. Since the attraction of molecules outside this circle is imperceptible, we may consider each molecule as attracted only by all other molecules in its circle. The molecular attractions on M_1 are equal on all sides and consequently neutralize one another. The upper part of the circle around M_2 ist out of the liquid and an equal and opposite part of the circle the stippled area contains molecules whose downward attraction on M_2 is not compensated by an upward attraction. The molecule M_3 is attracted downward only. This downward attraction on all molecules in a surface film equal in thickness to the range of molecular attraction pulls them closer together. The closer they are pulled together the greater their attraction for one another. Hence, the surface film of molecules under tension acts like a stretched skin, or membrane.

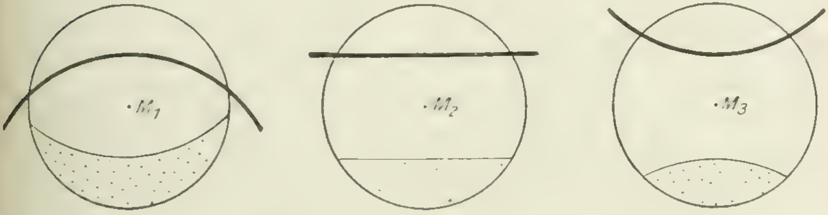
Fig. 1.



The magnitude of this surface tension depends on the magnitude of molecular attraction, which in turn depends on the chemical composition of the liquid, and also upon the temperature and pressure, since these affect the distance between the molecules. Surface tension also depends on the form of the liquid surface, as may be deduced from the following:

In Fig. 2 let M_1, M_2, M_3 represent three molecules each of which is the same distance beneath a liquid surface, represented by the heavy line above, which is plane over M_2, convex over M_1 and concave over M_3. Around each molecule the sphere of molecular attraction is represented by a circle. Very much of the volume of the sphere of M_1 projects above the surface, whereas less of the volume of M_2 and least of the volume of M_3 project. With the same reasoning as applied to Fig. 1 we may conclude that the downward attraction is greatest on M_1, less on M_2 and least on M_3. Hence surface tension is greatest on a convex surface, less on a plane surface and least on a concave surface. It should be remembered that every curved surface cannot be classed as convex or concave. The curved surface of a sphere or cylinder is convex when viewed from without and concave when viewed from within. However, the constricted portion of an hour glass when viewed from without appears concave along a meridian and convex along the equator. When the meridianal concaveness is as great as the equatorial convexness of the surface film of such a fluid body, no inward or outward force is exerted, as the result of surface tension. We have then what might be called a »surface of no curviture«.

Fig. 2.



If the liquid under consideration is in absolute vacuum the foregoing scheme is complete. but if molecules of any kind lie above the surface of the liquid they must be taken under consideration. If the liquid is covered by a second liquid, which for purposes of distinction we will call the medium, and the attraction of the molecules of the medium for those of the liquid is less than the attraction of the molecules of the liquid for one another, the downward tension on the molecules M_2 and M_3 in Fig. 1 and M_1, M_2 and M_3 in Fig. 2 will be decreased by the upward pull of the molecules of the medium filling the part of the sphere of molecular attraction which lies above the surface of the liquid. Hence the surface tension in all cases will be decreased.

For purposes of description one of the two liquids in contact has been called the medium, but it should be remembered that both liquids display surface tension, and what is usually called the surface tension of a drop of one fluid submerged in another, is realy the sum of the tensions of both fluids at their surface of contact. Therefore in considering the effect of curvature of a surface on its tension we must know which of the two liquids has the highest tension at their surface of contact, a fact which is ignored by Robertson (page 699). Where there is not an appreciable difference between these two tensions, the effect of curvature is negligible, since it would cause reciprocal changes in the two tensions. Therefore the best method of determining the effect of curvature in any given case is direct experiment, and this gives a method for determining which of the two liquids has the greatest surface tension.

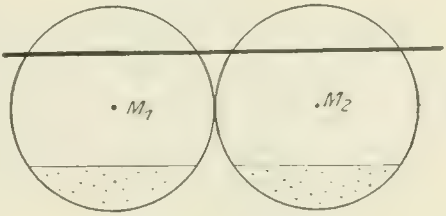
If there is dissolved in the liquid a substance whose molecules are attracted by the medium more strongly than the molecules of the solvent are attracted, the molecules of the solute that lie in the surface film will increase the upward pull of the medium and hence decrease the surface tension of the liquid (and of the medium). Also the solute will become more concentrated in the surface film than in the interior of the liquid. If the medium attracts the molecules of the solute less than the molecules of the solvent, these latter will tend to displace the molecules of the solute from the surface film. But those molecules of the solute, which by their kinetic energy force their way into the surface film, will raise the surface tension. Therefore, the raising of surface tension by dissolved substances requires the introduction into the surface film of (kinetic) energy from without. This is a fact which is entirely neglected by Robertson on page 696 in which he says in criticism of my work »without the introduction of energy from without, a phenomenon which is thermodynamically impossible«.

In so far as molecular attraction determines solubility (and we will assume that it is one factor in solubility) we may conclude that, if the molecules of the liquid are attracted by those of the medium as strongly as or more strongly than by one another, the liquid will mix with the medium and there will be no surface for consideration. Also if the molecules of a solute are attracted more strongly by those of the medium than by those of the solvent, the solute will diffuse into the medium. (Our proof of this is based on the assumption that molecular attraction is a factor in solubility. It is important to note that Willard Gibbs has furnished thermodynamical proofs of essentially the same conceptions and they have also been substantiated by considerable experimental evidence.)

A gaseous medium behaves in the same way as a liquid medium save for the fact that its molecules are so widely dispersed that its effect upon a liquid surface film is less marked. Hence the surface of a dense and poorly volatile liquid in contact with air always has a high tension.

In case of a solid »medium« the surface film loses its similarity to a stretched skin by being attached to the solid. Also, there is perhaps another peculiarity. Often liquids do not dissolve or are not absorbed by solids, even though the molecules of the liquid are attracted by those of the solid more strongly than by each other (adhesion). In the latter case, the molecules in the surface film of the liquid would be attracted more strongly toward the solid than toward the remainder of the liquid and hence be brought closer together and form a surface tension film. Also, if molecules of a solute are attracted by this solid more strongly than by the molecules of the liquid, they would become more concentrated in the surface film (adsorbed) and yet increase the surface tension.

Fig. 3.

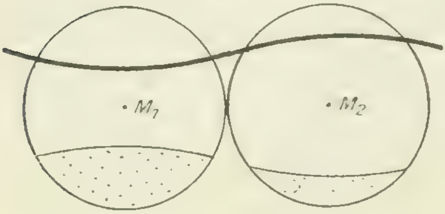


In any continuous surface of uniform curvature, if the surface tension is not uniform, surface movements will result. If the tension is increased over any area, that area will »contract«; consequently, if the tension is decreased over any area the pull of the remaining surface will cause the area of less tension to spread or »expand«.

This self evident fact may be analysed as follows: let the heavy horizontal line in Fig. 3 represent the upper surface of a liquid, and M_1 and M_2 two molecules at equal distances below the surface, and the circles about them the spheres of molecular attraction. Both M_1 and M_2 are attracted downward by a force equal to the attraction of the molecules in the stippled area of its sphere. However, suppose by a local decrease in temperature the molecules around M_2 are brought closer together and hence the number of molecules in the sphere of M_2 increased (causing increase of surface tension), then molecules from the adjacent regions are attracted toward the sphere of M_2 and the surface above M_2 is raised and made convex, while the surface above M_1 is lowered and made concave, as in Fig. 4.

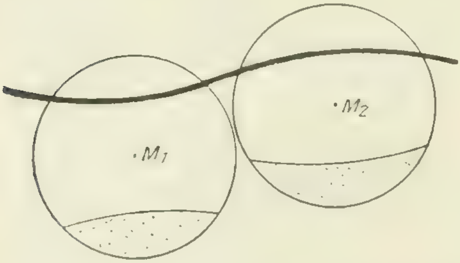
Now M_2 is pulled downward by the molecules of a smaller (stippled) region than M_1, but they are more crowded. Suppose the downward attractions on M_1 and M_2 are exactly the same. One might suppose that the difference in surface tension is also equalized, and ROBERTSON, on page 699, falls into this error (see his Fig. 2). However, M_1 is now nearer the surface than M_2 and a new figure must be drawn in which they are equidistant from the surface (Fig. 5). It may readily be seen by inspecting Fig. 5 that the surface tension is not equalized but that the difference is increased, and the surface tension cannot be equalized until the molecular attraction per unit volume is equalized. In other words, surface movements will continue until the chemical composition and temperature of the surface are uniform. (Whereas Figs. 3 and 5 serve to analyse surface movements, they do not apply to changes in curvature resulting from such movements, since the internal pressure or the fluid is left out of consideration.)

Fig. 4.



These surface movements, by friction on the adjacent fluids, cause more general movements. In case of a drop suspended in a liquid medium, spreading of the surface of one polar area will cause a vortex movement in the drop and another in the medium. Because of the inertia of the medium, these movements would lead to locomotion of the drop. (ROBERTSON neglects this factor in his erroneous consideration of locomotion of the *Amœba*, page 705.)

Fig. 5.



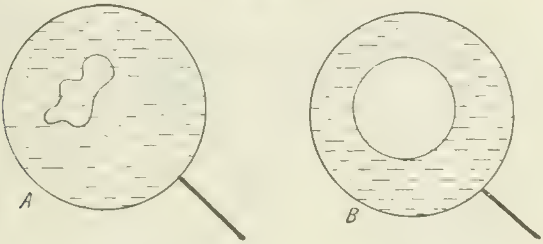
Since the surface film acts as a stretched skin it exerts no force perpendicular to the surface if that is plane. However, if the surface is concave, an upward pull is exerted and if it is convex a downward (inward) pressure is produced on the liquid beneath. The pressure within a spherical drop may be calculated as follows: Let R equal the radius and P the internal pressure produced by the inward pressure of the surface film. If the sphere is divided into two hemispheres by a rigid plane partition, the area of this partition will be πR^2 and the downward force on the upper surface of the partition, $\pi R^2 P$. The film meets the partition at right angles along the circumference of a (great circle), whose length $= 2 \pi R$. Hence if T is the surface tension of the liquid, the upward force exerted by the surface tension on the partition is $2 \pi R T$. Hence, since this upward force must be equal to the downward pressure, we have:

$$\pi R^2 P = 2 \pi R T$$

$$P = \frac{2\,T}{R}.$$

Surface tension may be illustrated by the following experiment. A wire ring is dipped into soap solution and withdrawn, so that the hole in the ring is closed by a liquid film. On this film a loop of thread is laid (Fig. 6, A). The film is then punctured within the loop and the film outside the thread pulling in all directions toward the wire ring, stretches the loop into a circular form (B).

Fig. 6.



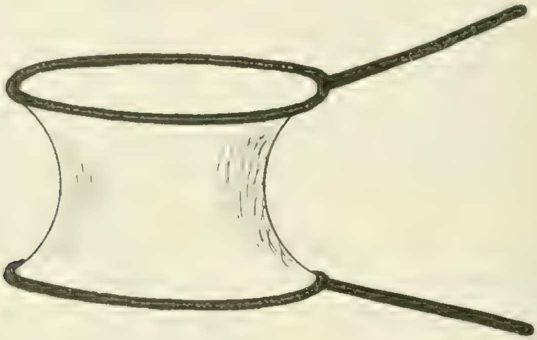
The spherical form of drops of a liquid is due to the tension of the surface film which acts as a stretched skin.

Surface tension of a liquid may be measured in the following manner: The liquid is sucked up into a pipette, the other circumference of whose mouth is measured in centimeters. The fluid is allowed to flow out slowly until a drop falls off. The drop is weighed in grams. The weight divided by the circumference is the surface tension, since a surface film, the length of whose cross section equals the circumference, supports that weight. If the measurement is made at the Earth's equator the above quotient may be reduced to dynes by multiplying by 978.

A »surface film of not curvature« may be illustrated as follows: Take two circular wire rings, place one on another and dip them in soap solution. While drawing them out of the solution separate them keeping them parallel so that a film stretches from one ring to the other but does not close either ring. A film shaped like the neck of an hour glass is produced (Fig. 7).

The reduction of surface tension by a solute may be illustrated as follows: Two stender dishes are filled with water and to one of them a trace of alkali or soap is added. A mixture of 2 parts chloroform and 3 parts rancid olive oil is sucked up into a pipette. The mouth of the pipette is held beneath the surface of the water and the mixture allowed to flow out slowly and drop off. The drops formed in the alkaline water are smaller than those in the pure water. This is due to the fact that the alkali acts on the fatty acid in the oil, forming soap, which lowers the surface tension. The lessened surface tension supports a less weight of the mixture on the end of the pipette, and the drop falls before it reaches a large size.

Fig. 7.



It will be noted that the drops resting on the bottom in the pure water are approximately spherical in form because the high surface tension resists the action of gravity. However, the drops in the alkaline solution are more flattened because the surface tension is lessened and the resistance to the action of gravity is lessened. This difference is accentuated if, by holding the mouth of the pipette near the bottom, we cause drops of the same size to be formed in the two dishes. If we take a piece of linen thread .4 mm. in diameter, soak it in water and remove all air bubbles, then place it on one of the drops in the pure water, the high surface tension of the drop resists the force due to the weight of the thread, and the thread slides off of the drop. On repeating this experiment in the alkaline water, the thread presses on and indents the drop, and if the surface tension of the drop is sufficiently lowered, the thread cuts it in two.

Instead of adding the alkali to the water, ROBERTSON soaked the thread in alkali. He used drops not over $^1/_{10}$ cc. in volume and submerged them not more than 5 mm. beneath the surface and soaked the thread in an alkaline solution $^1/_{10}$—$^1/_5$ normal. In this way the surface tension was weakened just beneath the thread, and if the process is very rapid the drop might be cut in two without as much general flattening. Apparently my first attempts at repeating his experiment failed because the threads I used were not heavy enough to cut through the drop, for ROBERTSON says linen threads .2 mm. in diameter rarely cause division.

In an attempt to exclude the action of gravity ROBERTSON subsequently buoyed up the drop by saturating the lower layer of water with NaCl. He found that the alkaline thread sometimes cut through the drop, especially if the alkali was increased to a normal solution, but he adds: »The best method of performing the experiment is to lower an alkaline loop of thread into the water and draw it gently up through the drop«! It should be noted that although the drop does not rest on a solid surface, the action of gravity is not entirely excluded, for the drop rests on a layer of NaCl solution of greater specific gravity than itself.

Surface movements due to local changes in tension may be illustrated as follows: If a glass plate is dipped into 95 $^0/_0$ alcohol, on removal it will be covered by a film of alcohol due to the adhesion of the alcohol to the glass. If a piece of cotton wet with water is held near a portion of the film, the surface tension will be increased, due to the localized increase in the water content of the alcohol. The surface of increased tension will contract, therefore each molecule in the surface film will move toward the center of the area. The friction on this surface layer of water will carry along layers beneath. Therefore all of the alcohol except that in close contact with the glass will be displaced toward the center of the area, where the alcohol is heaped up and the surface made convex. If the cotton is held near the surface of a spherical drop of alcohol, the surface currents are the same, but the molecules that moved to the center of the area turn downward (inward) and a vortex is formed. The surface of increased tension becomes more flattened due to the increased downward (inward) pressure of the surface film.

If the glass plate is wetted with water, and a rod wet with ether is brought near the center of the film, the ether vapor reduces the surface tension and the affected surface film spreads (as can be seen if powder is dusted over the surface). This spreading is caused by the pull of the surrounding surface film of high tension, which in turn is fastened to the glass at the periphery of the wet surface by adhesion. The water carried away from the center is temporarily banked up all around the edge of the affected area, and if the action is very violent, the center will become dry. This experiment is misinterpreted by ROBERTSON on page 705. If the ether is held near the surface of a spherical drop of water the affected area will be bulged out instead of sunken in, though this experiment is difficult to perform and observe in air.

The pressure in a drop due to the tension of the surface film may be measured as follows: Water is sucked up into a vertical capillary tube and allowed to drop out until equilibrium is reached (a source of error arises in the final breaking off of too large a drop), and the height of the meniscus in the tube noted. A dish of water is then raised from beneath until the surface of the water in the dish touches the drop. The meniscus immediately falls. The meniscus is higher while the drop hangs from the lower end of the tube because the surface tension of the drop causes pressure within the drop which is transmitted up the tube and raises the meniscus. The pressure of a column of water equal in height to the difference in level of the meniscus represents the pressure within the drop.

It is not my ambition to exactly imitate cell division with a model, but an analysis of the following experiment may throw some light on what hapens when a cell divides. A stender dish is half filled with distilled water and a funnel inserted so that the stem reaches the bottom. A saturated solution of pure NaCl in distilled water is poured very slowly into the funnel so that the salt solution comes to lie beneath the pure water without much mixing. About 1 cc. of the chloroform-oil mixture is poured in, so that it forms a drop suspended in the liquid. Two pipettes are filled with $^1/_5$ normal NaCl solution and quickly introduced into the water, and the alkali allowed to flow gently against opposite poles of the drop. If the alkali reaches both poles at the same time and in the same quantity, the drop elongates along the polar axis and usually constricts into an hour glass shape. It often divides into two at the constriction.

The conditions necessary for complete division are very limited, as is explained in the following analysis of the experiment:

The alkali reduces the surface tension at the poles and the pressure inside the drop causes a bulging of these regions. In Fig. 8 the surface film of low tension is represented by a light line and that of high tension by a heavy line. At the same time the polar surface films spread and the equatorial surface film contracts, producing vortex movements in the drop and in the medium as shown by the arrows (shown better in Fig. 9). These movements may be demonstrated by mixing a little soot with the oil and a few carmine grains with the water. The dashed arrows represent currents in the axis of the drop. The surface currents carry the alkali toward the equator and enlarge the polar regions of reduced tension. The rapid fall in the internal pressure caused by the rapid enlargement of the surfaces of low tension allows a constriction of the equatorial region, as in Fig. 9. The equatorial surface is not concave (as Robertson intimates) but remains (convex) until the meridional concaveness equals the equatorial convexness. When the surfaces of reduced tension have expanded until the area of high tension forms but a

Fig. 8.

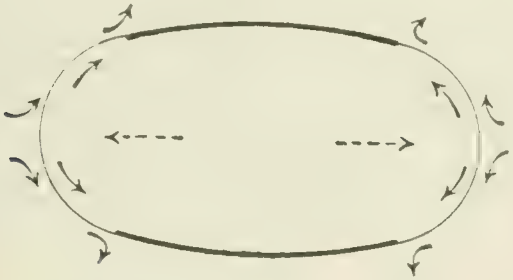
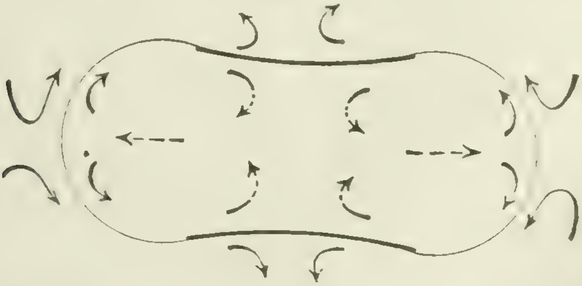


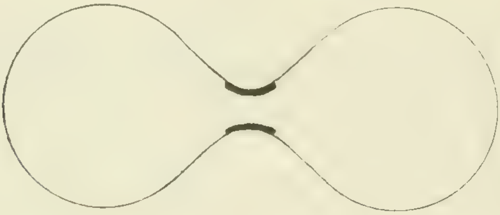
Fig. 9.



narrow band around the constricted equator, this band acts like an isolated band of a tension equal to the tension of its surface minus the tension of the surface of the polar regions. The entire force of this residual tension is exerted toward the division of the drop Fig. 10). However, the completion of the division takes time, and the band is rapidly reduced in width by the spreading of the alkaline surfaces. If the constriction is not completed before the alkali spreads over the whole drop, the drop returns to a spherical shape. Therefore it is unusual that a complete division of the drop occurs[1].

If a substance which increases the surface tension is liberated along an equatorial band, division will occur. However, a demonstration of this would be difficult for the following reasons. it would involve an increase in the sum total of surface tension, and since the surface is increased by division, a double increase of surface energy would have to be brought about, and would involve a double amount of work to overcome the inertia of the system. This work would have to be expended in such a way as to increase the surface tension i. e., by locally decreasing the temperature, or the electrical polarization, or the concentration of a substance which decreases the surface tension, or by increasing the concentration of a substance which increases the surface tension. A great difference in temperature or pressure is necessary for a small difference in surface tension, and the mechanism of maintaining the equator at a lower temperature or higher pressure has not been devised. Whereas some dissolved substances slightly raise the surface tension of water, they affect the specific gravity in a more marked degree and inter-

Fig. 10.



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[1] Failure to perform this experiment is due to lack of dexterity. The smaller the drop the relatively greater the surface tension but the greater is the dexterity required. Pipettes with capillary openings are more easily controled, and the concentration of alkali may be increased to compensate for reduced quantity, but it should not be heavier than the salt solution.

fere with the experiment. Dissolved substances which markedly raise the surface tension of liquids that could be used in a model, have not been found. If we start out with an oil drop in soapy water, the soap might be precipitated at the equator by a liberation of calcium, but the solid soap so produced would form a crust on the equatorial surface and interfere with its contraction. There remains the method of an equatorial decrease in electric polarization. A model on this principle has not been constructed. Electrical variations have, however, long been known to occur in living cells, and Miss Hyde has detected them in dividing eggs[1]. I have discussed the possibility of cell division being due to an equatorial increase in surface tension, following decrease in electrical polarization due to increase in permeability, in previous papers[2] however, I can no longer assume protoplasm to have an invisible alveolar structure).

Now that the whole subject has been reviewed, a direct consideration of Robertson's paper will be made. We have shown that in Robertson's experiment the oil drop is divided by the gravitation of the thread which has been soaked in an alkaline solution. The alkali reduces the surface tension, thus reducing the resistance to division, and thus allows division by a thread of less weight than would otherwise be necessary.

Robertson, on page 695, objects to the hypothesis that division can be brought about by an equatorial increase in surface tension, because the two drops resulting from division have a greater surface than the original drop, and if the surface tension is not decreased the potential energy of the surface is increased and work performed »without the introduction of any energy from without«. He forgets that work is done in increasing the surface tension of the equator. Robertson's assertion (page 699) that regions which are convex to the medium have relatively high surface tension is only true when the surface tension of the medium is less than that of the liquid in question, at the surface of contact, as has been pointed out above.

Robertson's argument on pages 699 and 700 falls flat when we note that M_1 and M_2 are not the same distance below the curved line *alrmd*.

---

[1] Hyde, Am. Journ. Physiol. Vol. 12. p. 241.

[2] McClendon, Am. Journ. Physiol. Vol. 27. p. 240; Biological Bulletin. Vol. 22. 1912. p. 158.

Robertson criticises my illustration of a rubber balloon. I make no defence of this because it was never supposed to be an exact model of cell division.

Robertson criticises my oil drop model of cell division. I have modified it as described above, to more nearly imitate cell divison. However, I make no claims for this model, except that the dynamics of this model throw light on the dynamical possibilities in cell division.

Robertson's illustration on page 705 of the effect of decrease of surface tension on the level of the surface, is not a general case, since the proximity of the glass plate interferes with the internal movements of the fluid. By increase in the thickness of the layer of water the result may be entirely changed.

Robertson's illustration on page 705 of a boy enclosed within a football, trying to progress by pushing against the inside, without success, reminds me of my youthful experiment with the opposite result. While enclosed in a large barrel I caused it to progress very rapidly. Surface movements could cause the locomotion of an amoeba suspended in water by driving the water in the opposite direction, in the same may that a steamboat progresses.

As to Robertson's hypothesis of the polar synthesis of nucleic acid in the daughter nuclei being causally related to cell division, I observed division of starfish eggs from which I had removed the nuclear material[1]).

Summary.

If a drop of liquid is suspended in a liquid medium, any area whose surface tension is reduced, spreads and protrudes, causing vortical currents, whereas any area whose surface tension is increased, contracts and becomes more flattened, causing a vortex in the opposite direction.

Division of a similar drop may be brought about by a condition in which an equatorial band has a higher surface tension than the remainder of the surface.

Robertson came to opposite conclusions, but five fallacies in his argument are pointed out above.

---

[1]) McClendon, Arch. f. Entw.-Mech. Bd. 26. 1908. S. 662.

Zusammenfassung.

Wird ein Flüssigkeitstropfen in einem flüssigen Medium suspendiert, so breitet sich jeder Bezirk mit verminderter Oberflächenspannung aus und treibt sich vor, indem er vortikale Strömungen hervorbringt. Dagegen zieht sich jeder Bezirk mit vermehrter Oberflächenspannung zusammen und flacht sich ab, unter Veranlassung eines Wirbels in der entgegengesetzten Richtung.

Teilung eines ähnlichen Tropfens kann durch Verhältnisse zustande kommen, bei denen ein äquatorial gelegener Streifen höhere Oberflächenspannung besitzt, als die übrige Oberfläche.

Robertson kam zu entgegengesetzten Folgerungen — im Vorstehenden sind aber fünf unrichtige Punkte in seiner Beweisführung aufgewiesen worden.

(Übersetzt von **W. Gebhardt.**)

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September, 1915

THE ORIGIN OF BLOOD AND VASCULAR ENDOTHELIUM IN EMBRYOS WITHOUT A CIRCULATION OF THE BLOOD AND IN THE NORMAL EMBRYO

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INTRODUCTION

The origin of blood presents an almost unique problem in embryology. First, on account of the fact that the initial blood anlage in many animals is contributed to by wandering cells. Second, owing to the establishment of an early flow or circulation of embryonic fluids before the blood corpuscles have arisen.

Soon after the cells and corpuscles are formed they are swept into this circulating current and carried to all parts of the body. In this way the blood cells become associated and mixed with numerous other types of cells, and it is difficult, if not impossible, to establish their true relationship with their surroundings. For the above reasons one is often ready to believe that many of the even careful and long thought out contributions to the development of blood are, after all, largely a matter of the author's own interpretation rather than a record of the actual processes.

The general current of opinion at the present time would seem to indicate that all blood cells arise from a mesenchymal type of cell. A number of very competent workers have described the change of this mesenchymal cell into a stem cell or mother cell. On one side from this mother cell are developed various leucocytes, which it is important to note always occur in an interstitial position, while on the other hand, this same general type of mother cell gives rise to other cells which later differentiate into typical erythroblasts, and finally erythrocytes which are always found to be located within the vessels. These so-called indifferent mesenchymal cells probably, from the evidence contained in the literature, do form blood cells, but to the discriminating reader the evidence is not at all convincing that both white blood cells and red blood cells really arise from one common mother cell or common embryonic anlage.

The possibility, and even probability, is certainly present that these so-called stem mother cells may in reality not all belong to one type, but are different and may already be destined to form either red cells or white cells. Yet on account of their wandering capacities as well as on account of the fact that the earliest blood cells are swept around in the circulating current they have become so mixed and confused that it is almost impossible to separate the cell groups or differentiate between them. One must in this connection, remember the fact that almost all authors have concluded that only red blood cells are formed in the blood islands of the yolk-sac in most vertebrates. All authors who have studied the development of the blood in Teleosts have invariably described only red blood cells as arising in the intermediate cell mass. No one has ever mentioned the presence of white blood cells in the true blood forming anlage of the Teleosts.

The monophyletic school really goes still further and not only claims that all types of blood cells arise from a common mother mesenchymal cell, but also that the vascular endothelial cell is likewise capable of giving rise to the various types of blood cells and is originally a cell of the same type as the stem mother cell. There are numerous descriptions and illustrations of the origin of blood cells from the vessel linings in the literature of the past twenty-five years, since Schmidt in 1892 described the transformation of individual endothelial cells into white and red blood corpuscles. Yet again, I believe that the really skeptical reader will not be at all convinced that such a thing ever takes place from the evidence presented in the literature, certainly not from any of the illustrations that have been made of this process.

No real vascular endothelial cell has been actually observed to metamorphose into a blood cell or to divide off another cell which forms a blood cell, and until such a direct observation is forthcoming one can only question the accuracy of the interpretation of the various observations up to now recorded.

The mesenchyme is a very generalized embryonic tissue and from it arise the various kinds of blood cells, endothelial cells, connective tissue cells, etc. There can be no doubt of the great genetic difference between blood cells and connective tissue cells, yet their parent cells are with our present methods indistinguishable. We may with equal justification go further and hold likewise that the cells from which the vascular endothelium, red blood cells and white blood cells arise are mesenchymal cells really differing in nature according to whether they will give rise to one or the other of the three cell types. Yet they may not differ from one another in any way by which we can at present distinguish them. If this proposition be true, or even if the weight of evidence lean in this direction, it is scarcely more justifiable to derive these completely different cells from a common mother cell than it would be to derive connective tissue and blood cells from a common mother cell.

Of course, we are only considering the mesenchymal cell just before its differentiation is to begin. Carried back further, no doubt, all the cells become more and more alike and possess more and more complex potentialities as is so thoroughly demonstrated by the numerous studies of cell lineage. In the beginning, of course, all cells arose from one single egg cell capable of giving rise to every tissue of the body, but after tendencies in differentiation have proceeded sufficiently far in the various cells some then form real mesenchymal cells. Later individual mesenchymal cells incline in certain directions and finally become incapable of giving rise to any other than the definite type of tissue or cells towards which their particular tendencies have directed just as certain endodermal cells become specialized to form the liver while others near by and at first indistinguishable from these give rise to the ducts and acini of the pancreas.

All of the vertebrate classes present these many questions of blood origin, etc., but the forms upon which this investigation has been conducted, the Teleosts, possess in addition many extremely interesting special problems. In all other meroblastic embryos the majority of the earliest blood cells arise in yolk-sac blood islands. Yet in many of the Teleosts there are apparently no early blood islands on the yolk, but all of the blood forming cells are contained within the embryonic body.

This intra-embryonal blood anlage has been frequently described by many authors as the "intermediate cell mass." The intermediate cell mass as has been suggested by Marcus ('05), Mollier ('06), and others, is really the homologue of the blood forming yolk-sac mesoderm in the other meroblastic types.

The bony fish is important as an object of study on account of the fact that so many of its organs and tissues arise in a way peculiar to the group and differing from the other vertebrate classes. The solid gastrular invagination described by Sumner ('00), the original solid condition of the central nervous system, the solid optic knob which changes into the optic vesicle, and in the present connection, the very particularly interesting solid cord of cells, the intermediate cell mass, which is to give rise to the red blood corpuscles of the individual make the Teleosts a group of great embryological interest.

The complexity of the problem concerning the origin of the various types of blood cells is then largely due to the migration and mixture of the cells involved. It is strange that up to now no investigator has attempted in an experimental way to analyze the situation. It would seem to be one of the most favorable problems for an experimental analysis, and in the end it is certainly an analytical problem.

If it were possible by any means to separate the anlage of the red blood cells from that of the white blood cells and prevent the flow of fluid in the embryonic body so that these cells would not frequently become intermixed, then it would seem possible to determine clearly the entire genesis of the various type cells. If all the types of blood corpuscles did arise from a common mother mesenchymal cell they should then be found in intimate association throughout all blood forming regions. Further, if the vascular endothelium really has blood forming power, it should be found that blood cells arise in any region of the embryo which possesses vessels lined by such endothelium.

There have been various experiments performed which have interfered more or less with the circulation of the body fluids of the embryo, but none of these experiments where aimed at a solution of the genesis of blood cells or have been used for such a purpose. Knower ('07) removed the heart anlage from early frog embryos and they continued to develop in some cases with almost no circulation. In other specimens there was a very feeble sluggish circulation due to the pulsation of the lymph hearts or of remnants of the heart which remained after the operation. The embryos were not particularly adapted for the study of the blood questions since some circulation always took place, and this no doubt was sufficient to contaminate the original sources of blood cells and so confuse the situation. Loeb ('12) has reported experiments on bony fish hybrids and embryos treated with certain chemicals in which there was a heart beat but no circulation. These embryos were, however, not studied for either blood or vascular genesis.

The first demonstration of the fact that the embryo could develop without the circulation of the blood was given by Loeb in 1893. He showed that Fundulus eggs developing in solutions of KCl had no heart beat and no circulation of the blood, yet some vessels formed. In 1906 the writer repeated this experiment and confirmed Loeb's results entirely, but found that the vascular system and general development of the embryo was extremely abnormal and was hardly reliable for conclusive studies on the origin of special tissues.

With these experiments in mind, and appreciating the problems indicated above regarding the origin of blood as well as vascular endothelium, I have undertaken an extensive experimental analysis of this subject in conjunction with a careful systematic study of the histogenesis of the blood and vessels in normal embryos. The results of the experimental study which has been carefully followed during the past three years are presented in the following pages of this paper.

METHODS OF EXPERIMENT AND MATERIAL

Six years ago, while studying the influence of alcohol and various anaesthetics on the development of Fundulus embryos, I noticed that many of these embryos had a feeble heart beat, but no circulation of the blood. At that time, particular attention was given to a study of the defects of the central nervous system and of the organs of special sense and no attempt was made to investigate thoroughly the conditions present in other tissues and organs of the body.

During the last few years, special attention has been devoted to the study of these embryos without a circulation of their body fluids with the main object of analyzing as completely as possible, the origin and subsequent development of the heart, vessels and blood. All of the experiments have been repeated through three summers in the Marine Biological Laboratory at Woods Hole. It has been possible to produce embryos that were almost normal in all particulars yet in which the blood failed to circulate on account of the fact that the heart was blind at one or both ends or disconnected at the venous end or finally was a completely solid cord of tissue.

The embryos were studied in life and the particular individuals which had been so observed were fixed and finally studied in microscopic sections. The observations made on the experimental embryos have in all instances been completely checked and controlled by careful detailed study of the blood and vessels in normal embryos.

The experiments have been performed on two species of Fundulus, heteroclitus and majalis, and the results are practically identical for both. The eggs were stripped from the female into small dishes containing no water and fertilized by milt pressed from the male. About fifteen minutes after the application of the sperm water was added to the eggs. In this way one gets a very high percentage of fertilized eggs, while fertilizing the eggs under water or adding sperm to a dish of water gives a much poorer result. The fertilized eggs are then divided into groups so that the experiment and control are all from similar sources. The eggs just before dividing into the two-cell stage were introduced into solutions of alcohol.

The solutions which gave the most favorable results were prepared in the following way: 50 cc. of sea-water was placed in each dish and to this was added respectively, 1.5 cc., 2 cc. 2.3 cc., 2.6 cc., 2.8 cc., 3 cc., 3.5 cc., of 95 per cent commercial alcohol. The eggs remained in these solutions for twenty-four hours, after which time the solution was renewed. After forty-eight hours the eggs were removed from the alcohol solutions and placed in pure sea-water.

To give a general idea of the way in which the embryos developed in these different grade solutions of alcohol, I may cite some of the details of one experiment. After forty-eight hours many eggs are dead in all the solutions. The dead eggs are thrown out. When seventy-two hours old many others are dead in the solutions with 1.5 cc., 2 cc., 2.3 cc., and 2.6 cc., while all but one individual had died in 2.8 cc. It should be added here that about seventy-five eggs are placed in each dish. From the 1.5 cc. solution sixteen are alive at seventy-two hours, several with various eye defects, the hearts are beating but contain colorless fluid and the only trace of red blood color is in the intermediate cell mass and caudal vein. Several others of this lot have a full circulation with corpuscles in the current. From the 2 cc. solution one has a feeble heart beat but no blood is visible and there is no circulation; some embryos have no circulation but blood is present in the posterior end of the body, while many have blood circulating with a strong heart beat.

This brief reference to the notes of the experiment show that such doses are just on the border line of effectiveness since some individuals in the solutions are not able to develop a circulation, while others with a higher degree of resistence do develop a more or less normal circulation. It is very important in such experiments to use these threshold doses since they are the least injurious possible to give the desired result. In this way one gets individuals which have no circulation of the blood, and, therefore, in which the blood anlage develops and remains in its permanent position, without having any serious defects or abnormalities in the general body tissues of the embryo.

From a study of such specimens carefully controlled by a study of normal individuals, one is fully justified, I believe, in drawing final conclusions as to the significance of the developmental processes taking place. It cannot be argued so far as the blood anlage is concerned that the conditions recorded are pathological or other than those which would occur in a normal genesis of the blood except that it never circulates.

Embryos which are intended for microscopic study have been prepared in the following manner: The eggs are placed in picro-acetic (saturated aqueous solution of picric acid and 5 per cent glacial acetic) from thirty to forty minutes, then put into 70 per cent alcohol. This is frequently changed in order to wash out the picric acid. After they have been about one-half hour in the 70 per cent alcohol, the egg membrane is removed with fine dissecting needles. This is the most favorable time for removing the membrane. If the eggs have been left for a long time in the alcohol, the membrane is more difficult to remove and the embryo is brittle and more liable to injury.

After removing the entire membrane, the yolk-sac is then punctured at its ventral pole and the yolk mass very slowly and cautiously removed from the sac. To remove the yolk mass, requires a great deal of practice and extreme care in every case. It should be done with the use of a binocular microscope so that the operator can be certain not to tear or destroy the yolk-sac or injure the delicate heart lying close above the yolk. After a great deal of practice it is possible to remove the yolk from a number of embryos and leave the yolk-sac in perfect condition with the heart and pericardium practically undisturbed. In the great majority of cases, however, it is generally impossible to completely remove the yolk. It is usually necessary to remove the yolk on account of the fact that when the eggs are imbedded in either paraffin or celloidin, the yolk becomes so hard that it often breaks the sections and makes it very difficult to get a complete or perfect series. When the yolk-sac is punctured one-half hour after having been in the 70 per cent alcohol, the yolk material is in a gummy or viscid condition and is more easily removed than at any other period tried.

After having removed the egg membrane and the yolk, the embryos are then allowed to stand twenty-four hours in 70 per cent alcohol when they are changed to 80 per cent to be kept until the time of imbedding. The embryos are imbedded in paraffin and cut in serial sections from five to ten micra thick. They are stained in hematoxylin and eosin and extracted or carefully differentiated so as to bring out a clear stain of the blood cells and tissues. A complete series of these embryos have been made from a time before the appearance of blood up to sixteen days old, the normal embryo hatches and becomes free swimming at about twelve days.

As mentioned above, a similar series of normal embryos have been prepared and used for comparison with these non-circulating individuals.

In order to be certain of the final developmental product of the blood cells of the fish, numerous smears have been made from various tissues and from heart blood taken from the adult Fundulus. In these smears one finds the various types of white blood cells and the ordinary red blood corpuscles of Teleosts.

THE STUDY OF LIVING EMBRYOS WITH AND WITHOUT THE CIRCULATION OF THE BLOOD

1. Normal development up to the establishment of a circulation

The rate of development of Fundulus embryos is very variable, differing at different periods of the breeding season, and also differing in groups of eggs from different individuals.

When twenty-four hours old, the germ ring has descended almost to the equator in the most rapidly developing individuals. In others the ring is only one-third way over the yolk sphere. The embryonic shield and the first line indicating the position of the embryo's body is now to be made out. At forty-eight hours, the yolk sphere is completely covered by the ectoderm, the embryonic body is well shown with the optic knobs projecting prominently and several somites easily distinguishable. The heart has not begun to pulsate and no blood cells or blood anlage are distinguishable in the living specimen. Very soon after this time, or at least by sixty-eight hours, there are about ten somites present and collections of cells on the yolk-sac are the first indication of blood islands. No pigment cells have formed up to sixty hours.

At seventy-one hours pigment cells are recognizable but the blood islands are not yet colored and are sparsely arranged over all the yolk region except the anterior half. Near the lateral borders of the embryo and on the posterior yolk surface the islands are most abundant. At this time there is still no visible heart-beat or circulation to be seen with the high power microscope. At seventy-five hours the pigment particles are just beginning to show in the chromatophores. A well formed vesicle is clearly seen at the posterior end of the embryo from forty-eight to seventy-two hours and older. This is the so-called Kupffer's vesicle, and it, like the pericardium, becomes greatly distended by an accumulation of plasma in those individuals which have no circulation.

Embryos with fourteen somites may still have no heart beat and no circulation of the blood. Any later than this, however, all normal embryos establish a heart-beat and a circulation of colorless fluid, there being no blood cells present in the initial circulating medium. Very soon after the circulation is established at first a few but quickly many blood cells are added to the stream. This is merely an abbreviated summary of the development of the Fundulus embryo up to the time of the establishment of the heart-beat and circulation, but as stated above, the rate is variable and it often happens that an embryo of seventy-two hours has already established a vigorous circulation and the plasma is loaded with well formed blood cells.

2. *History of experimental embryos to the time when a circulation should begin*

In the experimental embryos development proceeds more slowly than in the normal. The plasma which should circulate in the vessels accumulates in the sinuses over the yolk and finally seems to collect in great amount in the pericardium, the lateral coelomic cavities and in Kupffer's vesicle, so that these spaces become hugely distended and appear as great sacs or vesicles of colorless watery fluid. The excessive presence of this fluid in the pericardium seems to exert a mechanical effect which tends to separate the head of the embryo an unusually great distance from the yolk mass and thus stretches the heart out into a long string-like cord, passing from the embryo to the surface of the yolk.

This pushing away of the head from the yolk is very well indicated in figures 15 to 20, which show various types of hearts found in these embryos during later stages. The stretching or pulling out of the heart may possibly be the cause of the failure to develop its proper connections with the veins, or in some cases to establish and maintain its lumen. On account of these mechanical deficiencies in the heart, we find that it is incapable of propelling the body fluids and establishing the circulation of the blood. The fluids thus accumulate in the large sinuses or spaces, in most cases the coelomic spaces and in the case of Kupffer's vesicle in an endodermic or endo-mesodermic cavity.

The red blood cells become evident after the fluid accumulation has partially taken place. They are always seen to originate in definite localities and are never found in any place very distantly removed from these localized regions unless a partial circulation or accident of some kind has occurred. Although red blood cells in many places arise from wandering cells the blood cells themselves have little capcity to wander.

3. Early formation of blood cells in living embryos

a. Intra-embryonic blood cells: The chief place of blood cell formation is the intermediate cell mass which extends from about the level of the anterior portion of the kidney back posteriorly to behind the anus and well into the tail of the embryo. This is the principal blood mass, but in addition to this, there are present in all of these non-circulating individuals small blood islands over the posterior and ventral yolk regions. These blood islands are also present on the yolk of normal individuals but in these the islands are swept away when the circulation begins.

b. Yolk-sac blood islands. A number of observations were made on the embryos which failed to develop a circulation and also on normal embryos to determine the significance and relationships of the blood islands on the yolk as far as was possible in the living individuals.

In one experiment when the embryos were seventy-two hours old, or just about the time that the heart-beat was beginning in many, it was found that although the plasma was circulating blood islands were present on the posterior yolk region. Ten embryos were selected which showed these posterior yolk blood islands and isolated to determine whether the blood islands would subsequently enter the circulation or what their fate would be. Ten other embryos with a feeble heart-beat but with no circulation yet also showing small posterior yolk-sac blood islands were isolated for comparison.

On the following day, nine out of ten individuals which had established a vigorous circulation had no blood islands remaining on the yolk. All of the islands had been taken into the circulation or vascularized, so that instead of blood islands there was now a network of vessels over that portion of the yolk and the blood cells had entered the current. One of the ten embryos exhibited an abnormal arrangement of the blood vessels that was particularly instructive. On one side there was a large vein running from the embryo out onto the yolk and on this side all of the blood islands had disappeared forming a network of vessels which conducted the blood to the venous end of the heart. On the other side of the specimen there seemed to be a suppression in the development of vessels near the embryo. The islands were still in the same condition they had been on the previous day except that the cells composing them had become much redder so that there was now no doubt whatever that they contained erythroblasts and early erythrocytes.

The ten embryos which had no circulation on the previous day were now found to present the following conditions: Two had established a perfectly free circulation and all of the blood islands had been swept away. In two other individuals circulations were established in an abnormal manner so that many blood islands still remained and presented a bright red appearance. Six of the ten specimens had no circulation whatever and the entire arrangement of blood islands over the yolk was in exactly the same condition as on the day previous, except that the blood cells were now much redder in color.

During the course of the experiments similar tests and observations have been repeated four times. In each instance isolated groups of embryos showing yolk-sac blood island were selected and examined in order to ascertain on the following day the fate of these islands. In every case my experience was identically similar to that just described. The islands on the yolk-sac are often very far distant from the embryonic body as is readily seen by reference to figures 21 to 24. There is no doubt that wandering cells migrate out on to the yolk surface at a very early period and here give rise to blood islands comparable to those formed in other meroblastic embryos. It must be recognized, as I shall bring out in a consideration of the microscopic study of these embryos, that the yolk-sac of the fish is not entirely comparable to that of all other vertebrate types, yet there are many observations on the early living embryos which have convinced me that mesenchymal cells do wander from the embryo to various parts of the yolk-sac. These cells occupy a position between the ectoderm and the periblast (periblastic endoderm?) just as the peripheral mesoderm would in other yolk-sacs. Some of the wandering cells are future pigment cells of either the red or black variety to be mentioned later, others future endothelial cells, but many at least are to give rise to future blood cells.

Therefore, in embryos at about the beginning of the circulation one finds two distinct blood regions: The major region and most evident is the intermediate cell mass of former investigators, and the second position in which the blood cells are seen is the yolk-sac blood islands. The earliest yolk-sac blood islands are very easily overlooked. The writer had examined these embryos in great numbers and studied them for sometimes before finally discovering the existence of the early islands. With a high power single objective binocular, however, after their location is known the observer is readily able to see the nuclei of these cells in the posterior region of the yolk-sac, and they may then be followed from time to time. After these observations there is finally no doubt that blood islands do form on the yolk-sac of the Teleost embryo but these islands are probably to be regarded as disconnected portions of the intermediate cell mass or blood anlage.

It is freely admitted that this yolk-sac island blood formation may not take place in all Teleosts. The early wandering cells that here give rise to blood islands may in reality be compared to the growing out onto the yolk of masses of cells from the intermediate cell mass as described by Swaen and Brachet ('99, '01). These cells in the species here studied may wander earlier and more freely than in the trout.

At any rate, as shall be brought out later, the ventral mesoderm of the yolk-sac in other vertebrates and the intermediate cell mass of the Teleosts are very closely related homologous portions of the mesoderm, if not one and the same thing.

4. The five-day embryos

The conditions which the embryos have attained five days after fertilization are illustrated in figures 1 to 4. In figure 1 a normal individual of this age is shown. The heart is slightly twisted but still more or less tubular. The vascular network on the yolk-sac is well established. The pigment cells are numerous but not yet fully developed and have not assumed an alignment along the blood vessels or taken on the usual embryonic pattern. The heart, of course, is pulsating vigorously and the blood current is easily seen both within the embryo and on the yolk-sac.

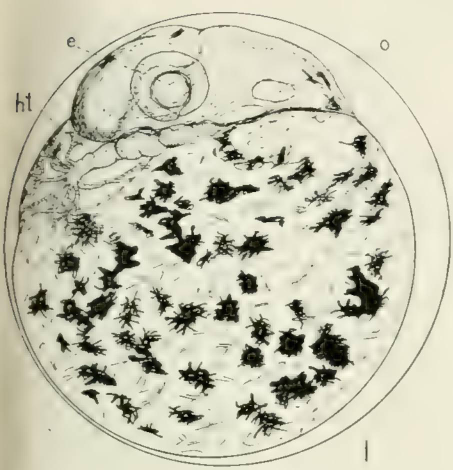
The three other figures in this group show different conditions of arrested development in individuals without a blood circulation. In figure 2 the pericardium is seen to be hugely distended so that the head is pushed or raised away from the yolk surface and the heart is stretched out into a long narrow tube extending from the ventral surface of the head to the sheer anterior surface of the yolk. This heart pulsates feebly and can be seen to contain a small amount of fluid which is churned up and down by the pulsations. None of this fluid, however, is ever pumped away from the heart. The pigment is much less plentiful than in the normal embryo of the same age, and the individual chromatophores are smaller in size than those of the normal embryo and have not sent out processes of any great length. No blood vessels at all are seen within the yolk-sac but very small scarcely noticeable blood islands are present on the posterior yolk region though not indicated in the sketch.

Figure 3 illustrates a more or less similar condition seen from a dorsal view. A small portion of the heart slightly projects beyond the anterior end of the head. If this egg were placed in lateral view, as was the case in figure 2, then the heart would be seen to show a similar condition, since it also was stretched out into a long narrow tube.

Figure 4 represents a very defective embryo. Specimens similar to this occur in great numbers in the stronger alcohol solutions. The bodies are very short since the descent of the germ ring over the yolk sphere is slow and at times incomplete, and the tail end of the embryo is often bifid or split giving a condition of cauda-bifida. At the anterior end, the upper right side of the figure, is shown the distended pericardial vesicle, and at the posterior end another large distended vesicle is in most cases the Kupffer's vesicle, but in some instances this is possibly distended spaces in the yolk mass just below the Kupffer's vesicle. These two sacs or spaces at the opposite ends of the body seem to be the places in which the non-circulating plasma most often accumulates to a great degree, in fact the accumulation of plasma is the actual cause of the exagerated condition of the spaces. In the individual illustrated by figure 4 the chromatophores are extremely small, but have arranged themselves to some extend so that they are very abundantly accumulated around the periphery of the Kupffer's vesicle while others have collected in the region of the pericardium. In the lateral yolk regions there are scarcely any pigmented cells present. All of these chromatophores, however, are small and contracted with very few processes of any extent.

In Fundulus embryos there are readily seen two distinct types of chromatophores. The one is a large dense perfectly black body with short broad processes. While the second is of a reddish color at first small and without processes, but later sending out very long graceful radiations which grow at the expense of the central mass until finally the whole chromatophore assume a moss-like branched structure, figure 6 shows both types well expanded.

Loeb ('93) at one time pointed out that these chromatophores migrate to the blood vessel walls and thus map the circulation



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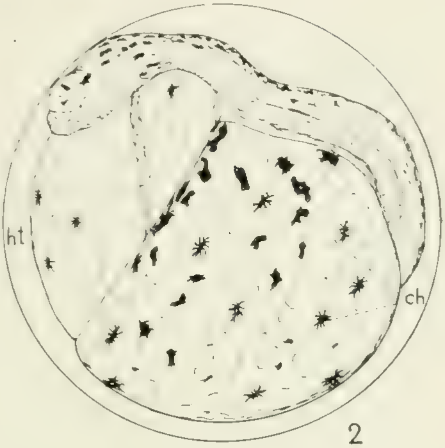

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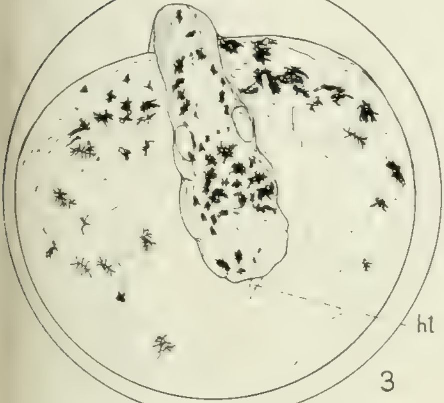

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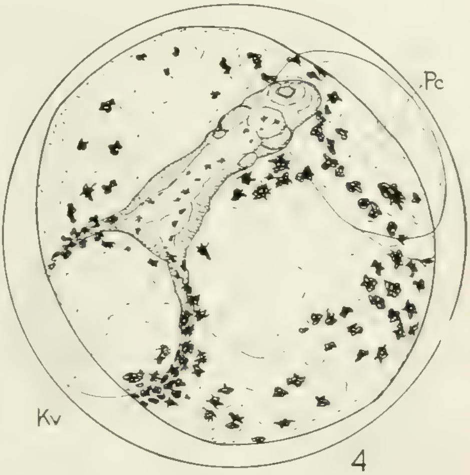

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Fig. 1 The head end of a normal Fundulus heteroclitus embryo from life, five days old; *ht*, the heart, showing its S-shape; *e*, eye; *o*, ear.

Fig. 2 From a living embryo of the same age, developed in a weak solution of alcohol; the blood does not circulate and the body is small; *ht*, the heart stretched into a straight tube surrounded by a much dilated pericardium; *ch*, small and unexpanded chromatophores.

Fig. 3 The head end of a five-day embryo without a circulation, from a weak alcohol solution; *ht*, the heart slightly projecting from beneath the head.

Fig. 4 A five-day embryo from a stronger alcohol solution; the eye is cyclopean, the posterior end of the body split, cauda-bifida, and no blood circulation. Pigment cells are collecting about the sinuses; *Pc*, distended pericardium; *Kv*, Kupffer's vesicle, also hugely distended.

on the yolk-sac of a normal embryo. While in embryos treated with KCl in which there was no circulation, the pigment cells failed to asume any definite pattern. They remained more or less indefinitely scattered over the surface of the yolk and the body of the embryo in no way tending to align themselves along the vessel walls. From this fact, Loeb concluded that it was probably due to some chemotactic reaction that the pigment cells lined up along the blood vessels when the blood began to circulate and the attracting substance was possibly the oxygen contained within the blood corpuscles. Observing the various individuals without a circulation which we shall here consider, it will be seen that the pigment cells have a strong tendency to migrate to any cavity filled with plasma or fluid, and it is not probable that this plasma or fluid contains any more oxygen than is present in the other portions of the yolk-sac or body. It would, therefore, seem more likely that some constituent of the plasma itself and not the oxygen contained within the blood cells was the stimulating principle which caused the migration of the pigment cells to the vessel walls.

5. The eight- and ten-day embryos

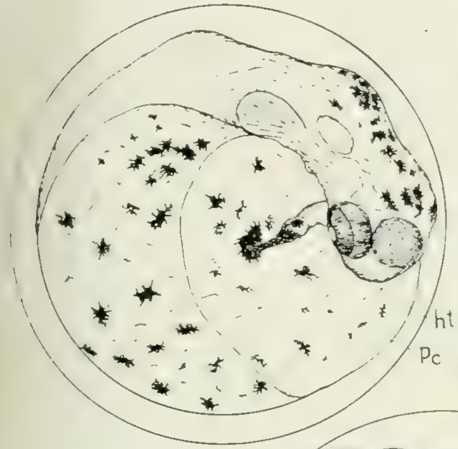
The next group of figures illustrates the advanced condition which the embryo has reached by the eighth day. Figure 7 represents a normal Fundulus embryo of this age. The body

Fig. 5 An embryo eight days old, without a circulation; the heart, *ht*, poorly developed, beats feebly twenty-eight times per minute, about one-quarter the usual rate; *Pc*, pericardium greatly distended with fluid; from a "1.5 cc. alcohol solution."

Fig. 6 Eight-day embryo without a circulation, *ht*; the heart dilated with plasma pulsates ninety-five times per minute: *RCh*, the red chromatophores beautifully expanded, but no vessels present on the yolk. *Coe*, the lateral coelomic cavity dilated with fluid; *icm*, the intermediate cell mass now a great string of red blood corpuscles.

Fig. 7 A normal eight-day embryo, the heart, *ht*; pulsating rapidly and the network of yolk vessels mapped out by the chromatophores.

Figs. 8 and 9 Two eight-day embryos without blood circulation; chromatophores unexpanded but collected on the heart, *ht*; the normal heart has no pigment cells on it; *Pc*, the dilated pericardium; *icm*, median mass of erythroblasts: *CV*, cardinal vein containing erythroblasts.



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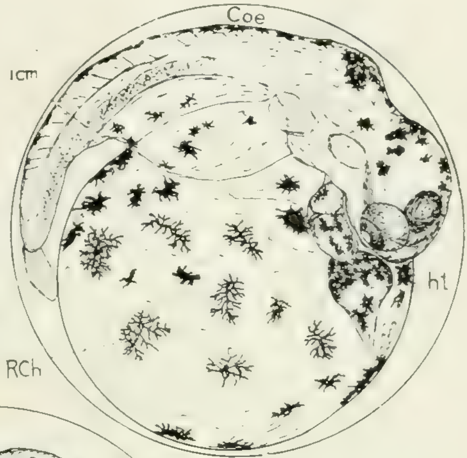

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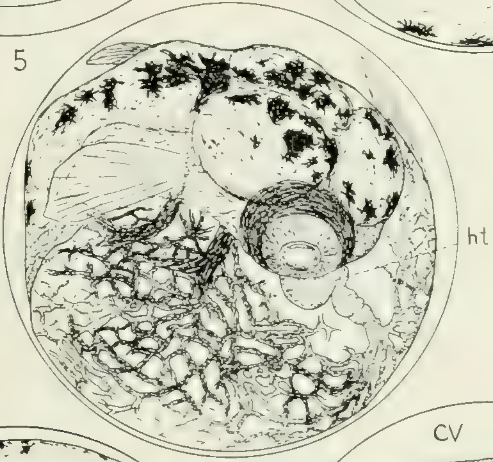

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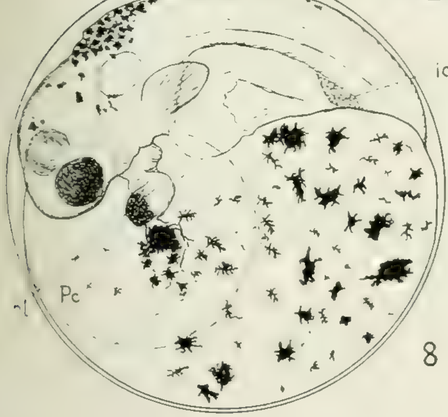

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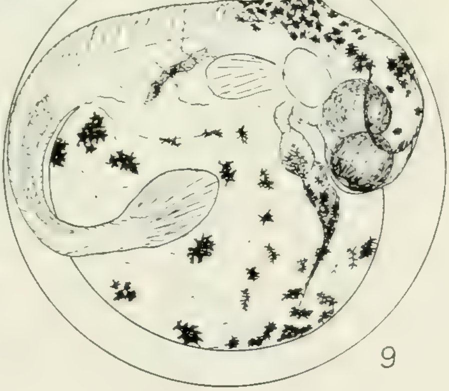

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is seen to be well developed, the fins are already capable of movement, and the brain and spinal cord are well shown and covered with the black type of chromatophores. The heart is seen to be more twisted than in the younger embryos and now occupies a position further under the head of the specimen. The network of vessels on the yolk-sac is beautifully mapped out by the arrangement of the pigment cells, largely the red type of chromatophore. It is to be especially noticed that pigment cells are never present on the heart of the normal embryo.

The other four figures of this group show individuals in which there was no circulation of the blood, although the hearts pulsated in a more or less feeble manner. In figure 5 the greatly distended pericardium is again shown, the heart is stretched from the head to the anterior surface of the yolk, and the lower part of the heart is completely sheathed with pigment. All of the pigment cells, however, are small and unexpanded.

In figure 6, the heart is very greatly distended and filled with plasma, yet it is apparently closed at one end since the plasma is churned up and down and never pumped out of the heart. In this case, there were several cells or particles suspended in the plasma contained within the heart, and these particles could be watched for long periods of time constantly moving up and down but never going out of their confined position. The pigment cells in this individual are greatly expanded, the red type chromatophores showing beautiful mossy processes. The lateral body cavities, *Coe*, or the coelomic spaces formed between the layers of the lateral plates of the mesoderm are greatly distended with plasma. A condition particularly noticeable in many such individuals. Red blood corpuscles are distinctly seen throughout the entire extent of the intermediate cell mass as indicated in the figure by the stippling in the posterior region of the body and the tail. The heart of this specimen is also richly covered with pigment and thus presents a striking contrast to the normal heart in figure 7.

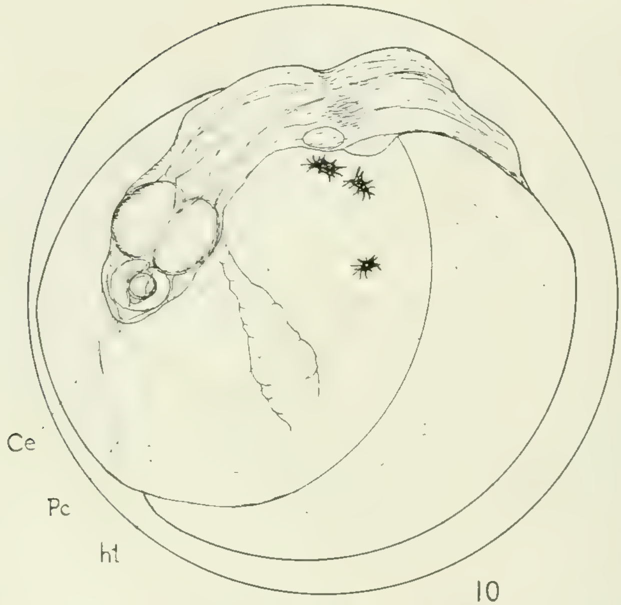
In figure 8 much the same condition is presented except that here again the pigment cells are still contracted. The pericardium, however, is distended and the heart is covered with pigment. The anterior end of the intermediate cell mass showing erythroblasts is just seen where the body of the embryo turns over the yolk sphere.

Figure 9 illustrates a lateral view of a small embryo. In all of these embryos in which the blood fails to circulate the fins are much smaller and less well developed than in the control, the entire body of the embryo is smaller and the whole appearance is that of a general developmental arrest, the rate of development being behind the normal. Yet such individuals have rather perfectly formed bodies, are capable of movement and seem in general to be very well developed, their only defect, so far as can be determined in many cases, is the absence of the circulation of the blood.

In figure 9 the heart is again sheathed with pigment cells, the blood cells in the intermediate cell mass are very distinctly present in the posterior body region, and in this individual a lateral vein in the position of the posterior cardinal also contains blood corpuscles. This appearance is seen in a number of individuals and may merely result from the fact that in these the intermediate cell mass is bilateral or split rather than entirely median in position. Such an explanation will seem probable, I think, after a consideration of the embryos in section.

Figures 12, 13 and 14 show three individuals of ten days old. These happen to be more or less abnormal. Figure 12 has very small eyes but the general body structure and shape are fairly normal. The pericardium is dilated, and the heart is small and pulsating feebly with a little pigment towards its aortic end. There is a great accumulation of pigment cells around the posterior region of the yolk sphere and near the distended Kupffer's vesicle in this embryo. Here again the cardinal veins are seen to be loaded with blood and only in the posterior body region do the two lateral masses come to unite into a median cell mass. Figure 13 shows much the same conditions, the heart is a mere filament indicated by the chromatophore along it.

Figure 14 gives a dorsal view of an embryo of ten days. The pigment spots are very few in number and the embryo has a pale



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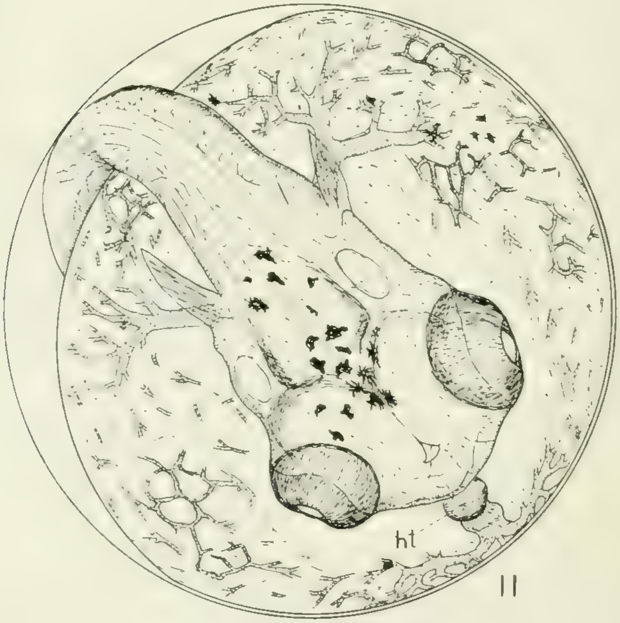

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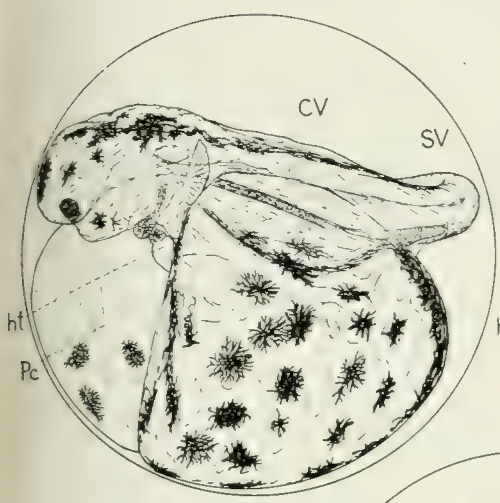

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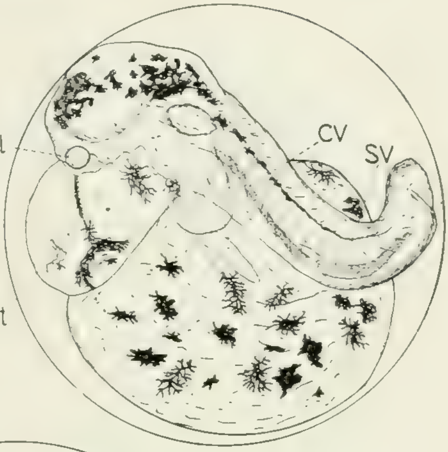

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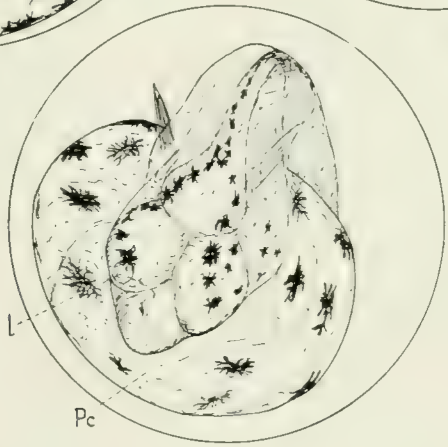

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Fig. 10 An eight-day embryo of Fundulus majalis from 2 cc. alcohol solution, showing a condition similar to the heteroclitus embryos; *Ce*, cyclopean eye; *Pc*, distended pericardium; *ht*, straight heart and no circulation.

Fig. 11 A normal majalis embryo of eight days with S-shaped heart, *ht*, and yolk vessels forming a net. Same magnification as the smaller heteroclitus embryos.

Fig. 12 A ten-day heteroclitus embryo, no blood circulation, chromatophores expanded and accumulated on posterior yolk region; *ht*, heart; *Pc*, distended pericardium; *CV*, cardinal vein filled with erythroblasts; *SV*, stem vein also full of red blood corpuscles.

Fig. 13 Embryo ten days old; *ht*, the heart a mere string covered with pigment; *l*, an independent crystalline lens; other lettering as in figure 12.

Fig. 14 View of head of ten-day embryo, no circulation, distended pericardium, *PC*; *l*, free lens.

appearance when compared with a normal individual, such as the one of eight days shown in figure 7.

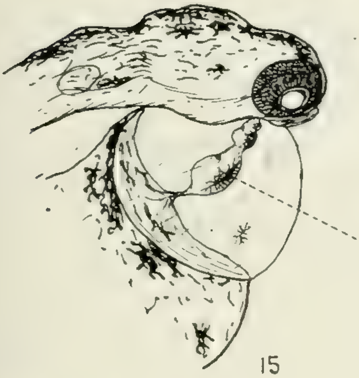
Figures 10 and 11 illustrates two specimens of Fundulus majalis drawn to the same scale as the previous figures. The egg of this species is considerably larger than that of heteroclitus, but its response to the experimental treatment is the same.

Figure 11 represents a normal embryo eight days old. It is seen not to be comparatively so far advanced at this period as is the heteroclitus, since its development is much slower and it requires from five to ten days longer to hatch. Very little pigment is present, yet the vessel net is well formed on the yolk-sac and the heart is distinctly seen to be more or less S-shaped.

Figure 10 is an embryo of the same age that had been subjected for forty-eight hours to a solution of 2 cc. of 95 per cent alcohol in 50 cc. of sea-water. Scarcely any pigment is present, the pericardium is typically distended and the heart is stretched into a long conical shape. No vessels are seen upon the yolk. The posterior end of the embryo is not shown in the figure, but in it could be seen early blood cells in the intermediate cell mass while a few small blood islands were present on the yolk-sac near the posterior end of the embryo.

After these eight- or ten-day stages very few changes of interest take place. The normal embryos hatch at from eleven to fourteen days as a rule, and become free swimming. The individuals without a circulation of the blood never succeed in breaking out of the egg membrane but may remain alive for twenty-five or thirty days in some cases, and almost all of them will live at least sixteen to twenty days. The red blood corpuscles are very distinctly noticeable in these older individuals and can be seen to remain permanently in their original places of origin. The intermediate cell mass may cease to be distinguishable, as was evident in the old specimens. This, however, is not due

Figs. 15 to 20 Living embryos sixteen days old, without blood circulation, showing variations in the pericardial distension, the position of the heads, and the perculiar heart conditions. These hearts, *ht*, all pulsate feebly and in several figures the slight lifting of the anterior yolk membrane is shown; this small membranous cone is rythmically raised with the pulsations.



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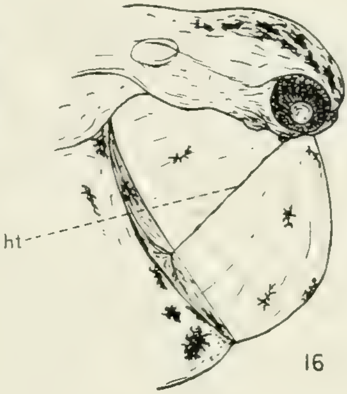

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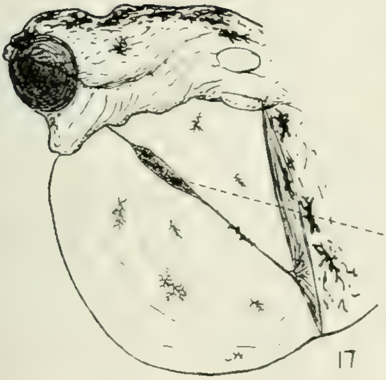

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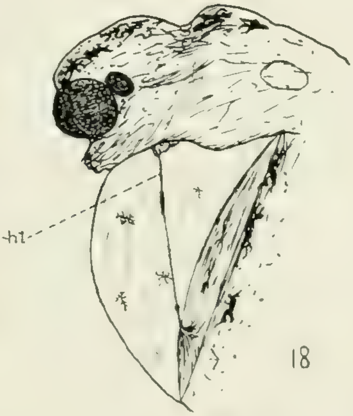

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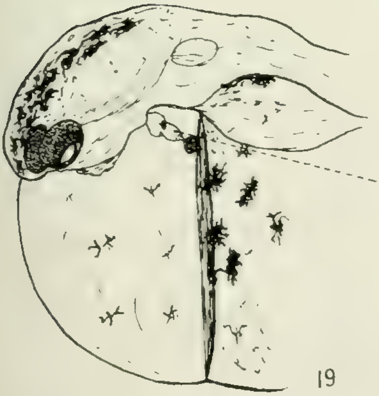

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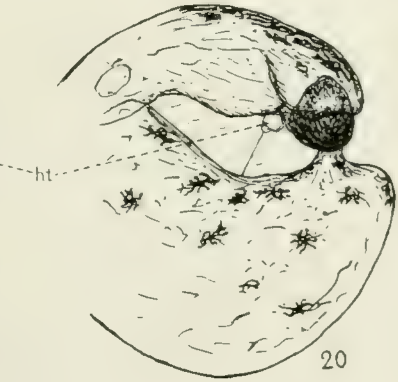

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to the blood cells having wandered away from the mass since they have largely degenerated *in situ* probably on account of lack of aeration. The blood islands on the yolk-sac maintain their red color for a much longer period of time, and they continue to present a pattern closely identical with that seen in the same individual during its earlier stages.

6. *Condition of the heart in old embryos without a circulation*

The conditions of the heart in some of these old embryos is well shown in the series, figures 15 to 20. These figures are from embryos of sixteen days old. The control specimens at this time would as a rule have hatched. In the sketches the peculiarly distended pericardium is strikingly shown. This great distention of the pericardium seems to have exerted pressure in such a way as to have straightened the anterior end of the embryo and lifted it well away from the yolk surface. The mechanical pull caused by the separation of the head from the yolk would seem to be largely responsible for the fact that the heart becomes stretched into a very much attenuated tube or string.

In the upper left hand figure 15, the heart is not so greatly stretched and the pericardium in this case is not distended so much as in the others. The upper right hand figure 16, and the two central figures, 17 and 18, show the pericardium distended to its utmost, and in these specimens the heart is pulled out into a mere string. Pigment cells seem invariably to wander along these string-like hearts, and they, therefore, stand out in the embryos as a black cord just as is indicated in the figures. The venous end of the heart which is connected with the yolk-sac is seen at each pulsation to lift slightly the yolk membrane in a cone-like projection from the surface of the yolk. As these hearts pulsate in their feeble fashion, one thus observes the yolk membrane as it is pulled up and down.

The two lower figures, 19 and 20, show other somewhat different conditions of the heart. The hearts are small and do not reach so far towards the ventral portion of the yolk.

There is an almost limitless variety of peculiarly abnormal hearts in these embryos and the six figures convey but a slight idea of the many very strange conditions which are presented.

7. *Development of the yolk-sac blood islands in life*

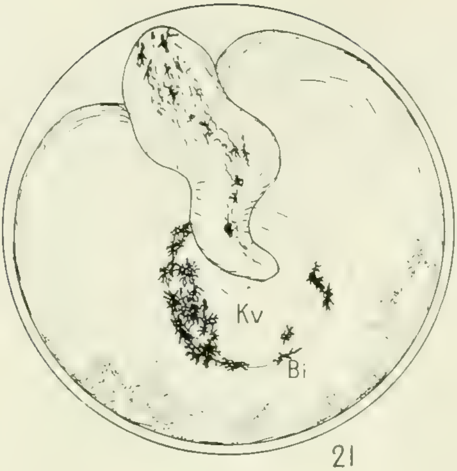
The blood islands in the living embryos, as was mentioned before, are quite difficult to see in the early stages. But a few hours before the heart begins to pulsate and the circulation bebecomes established they are very evident in the posterior ventral yolk regions. The arrangement of the blood islands display various patterns in different individuals in some being inconspicuous while in others an extensive network is present. These blood islands probably arise largely from wandering mesenchymal cells since the yolk-sac of the Fundulus embryo consists at first only of the yolk periblast with the ectoderm immediately above it. There is no true mesodermal layer to the yolk-sac and this mesenchymal blood formation on the yolk can, in all cases, be traced to the early wandering cells.

In figure 21 the posterior end of an embryo of ninety-six hours is shown. The Kupffer's vesicle, *Kv*, is dilated and pigment cells have accumulated around it. Immediately posterior to this are a number of blood islands indicated by stippling.

Figure 22 shows an embryo eight days old with the posterior ventral surface of the yolk well covered with blood cells. Erythrocytes are also seen in the intermediate cell mass. The blood in this embryo has never circulated and one can scarcely conceive that the blood islands on the extreme ventral surface of the yolk are due to the crowding out or pushing away of cells from the intermediate cell mass within the embryo. These cells are rather to be regarded as true yolk-sac blood islands which have arisen from early wandering mesenchymal cells probably in the beginning derived from same source as the intermediate cell mass.

Figures 23 and 24 show the caudal ends of two normal embryos of seventy-two hours. In these the heart has not begun to pulsate nor the blood to circulate, yet a distinct group of erythroblasts or early blood cells are seen already arranged on the yolk-sac in this posterior region.

In figure 24, it would look as though these cells had wandered out from and grouped themselves around the tail end of the embryo. At this period, seventy-two hours, the intermediate cell mass within the embryo is not visible in life.



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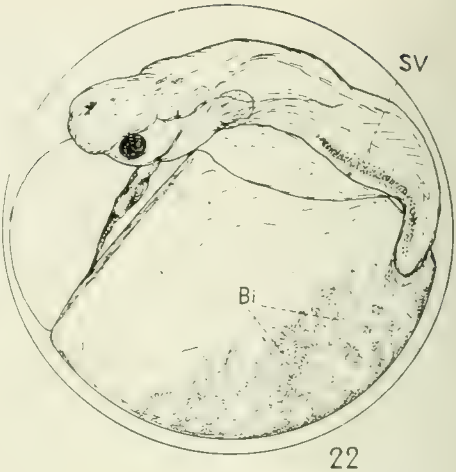

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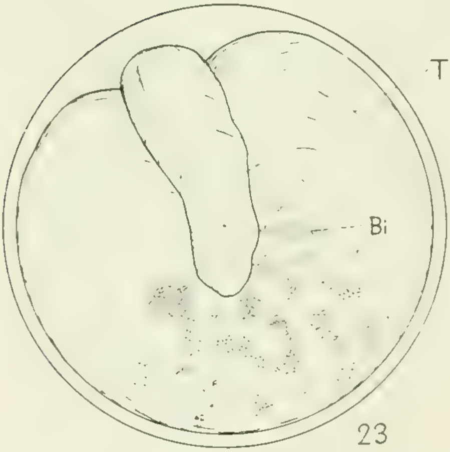

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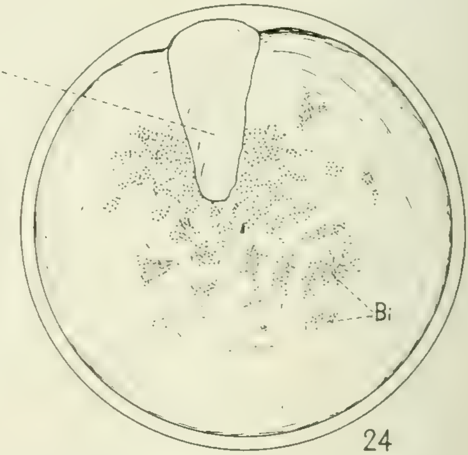

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Fig. 21 Caudal end of an embryo of ninety-six hours, without a circulation; *Kv*, the distended Kupffer's vesicle with pigment cells collected around it. *Bi*, yolk-sac blood islands on the posterior yolk region.

Fig. 22 Lateral view of eight-day embryo, without a circulation, showing red blood corpuscles in the stem vein, *SV*, and also masses of blood islands, *Bi*, on the posterior ventral yolk regions.

Figs. 23 and 24 Posterior veiws of two seventy-two hour embryos, without blood circulation. Cells are seen wandering out from the tail, *T*, region into the position of the peripheral mesoderm in most meroblastic eggs; these cells collect into groups and form the blood islands, *Bi*.

It may then be concluded from a study of the living embryos with a circulation and others without a circulation, that in the normal ordinary individuals as well as in those having their blood flow prevented, the origin and formation of blood in the bony fish occurs as follows: The chief source of origin of the erythroblasts is that so fully described by previous investigators as the intermediate cell mass, la masse intermédiare. This mass, according to Felix ('97), Swaen and Brachet ('99, '01) and others, arises from the median portions of the two lateral mesodermal plates, primary seiten-platten. These bi-lateral masses migrate towards the middle line and there fuse to form the intermediate cell mass or blood string. In the living embryo this very important mass of blood cells is readily demonstrated. It is usually median in position, but in many cases, as illustrated above, it may be double or bi-lateral, at least in its anterior portion. This bilateral arrangement may possibly be the result of a failure of the blood forming portions of the two lateral plates to move to the middle line and fuse to form a Stammvene, in other words, a type of arrest.

The second seat of differentiation of red blood cells which is distinctly shown in living embryos is to be found on the yolk-sac in the posterior and ventral region where numerous typical blood islands form and develop. All recent investigators of the development of the blood in Teleosts have denied the development of blood on the yolk-sac. Most of their investigations have been on the eggs of the trout, and it may be that in this group there are no blood islands. But in Fundulus we seem to have a transitional condition in which the yolk-sac islands have not been firmly incorporated within the intermediate cell mass but still remain out or wander out upon the yolk. At any rate, we must conclude that there is a secondary seat of red blood formation in Fundulus embryos, and that in life it presents the typical appearance of yolk-sac blood islands.

From a study of the living embryos, it is apparently impossible to determine whether all cells of these blood islands are only erythroblasts or of mixed types. This is, however, readily ascertained by a careful study of sections.

THE ORIGIN AND HISTOGENESIS OF VASCULAR ENDOTHELIUM AND BLOOD CORPUSCLES AS DETERMINED BY STUDY OF MICROSCOPIC SECTIONS

1. The structure of the heart in embryos without a circulation

The hearts of the embryos in which there is no blood flow have been described in the living in the preceding consideration, but when they are studied in section an additional number of very instructive points are brought out.

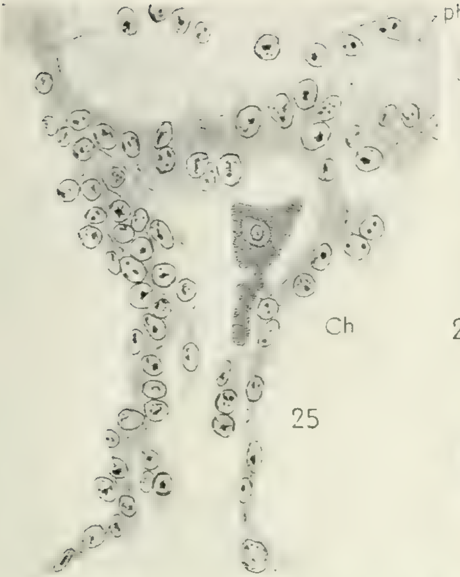
In the first place, the heart wall is usually very thin and not well developed. This is particularly true, in the long string-like hearts that are present in those individuals in which the pericardium is so greatly distended. In the group of figures 25 to 28, one sees sections of these hearts taken through various regions.

Figures 27 and 28 show sections through a long narrow heart. In figure 28 the myocardium is seen to be practically one layer of cells, and within this the endothelial lining is distinctly formed. No noticeable structural difference beyond slight variations in shape can be determined between the nuclei of the mycocardium and those in the endocardium. The myocardial layer is a thick more or less structureless cell mass while the endothelium is well differentiated into a thin single cell layer lining. This condition is found in a non-circulating embryo of four days old. Tracing the series towards the conus end of the heart, we find the arrangement indicated in figure 27. The myocardium is here also a thick layer of cells enclosing a distinct endothelial tube.

Fig. 25 Section through the heart of a four-day embryo without a circulation; Experiment 11, 1912, Embryo 6. Heart wall poorly formed; large chromatophore, *Ch*, in wall; *ph.*, pharynx.

Fig. 26 Section of a similar heart; Experiment 11, 1912, Embryo 2. The guide outline gives the general relationships of the heart. *MC*, myocardium; *EC*, endocardium; *Br*, brain; *pb*, periblast nuclei, and, *pbs*, periblastic material filling the heart cavity, *c*; red staining cell.

Figs. 27 and 28 Through the aortic end and figure 28 through the tube-like body of a similar heart; Experiment 11, 1912, Embryo 7. *Br*, brain; *ph*, pharynx; *EC*, definitely formed endocardium, endothelium; *MC*, myocardium. The nuclei of the endocardium and myocardium are indistinguishable except for slight differences in shape.



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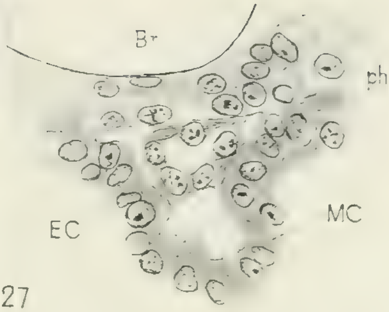

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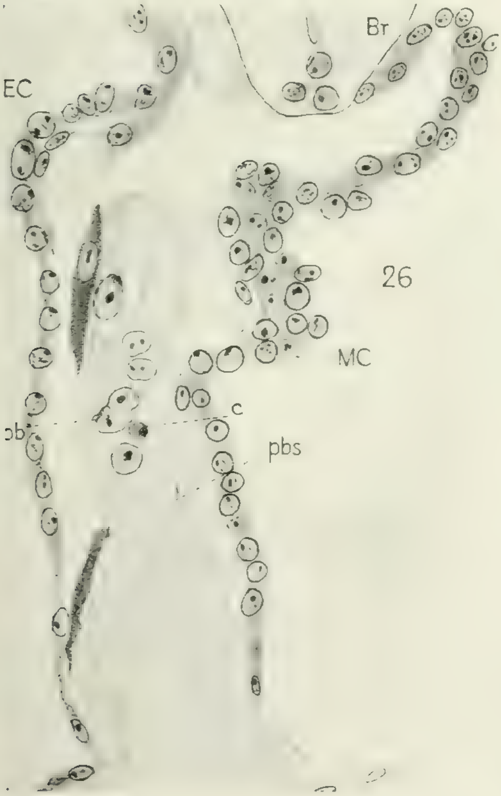

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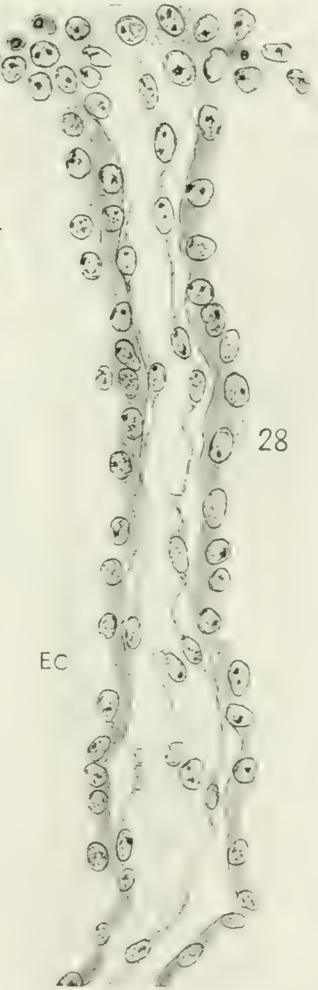

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Figure 25 is a section through another heart of the same age. In this a huge pigment cell is shown within the heart cavity. It is recalled that pigment cells were frequently seen to lie along these abnormal hearts while chromatophores were never present on the normal heart. The endothelium in figure 25 is more or less broken and the general condition of the heart is poorly developed.

In figure 26, a section is illustrated through a heart as it leads into the aortic arches. Here also large pigment cells are present. The endothelium is indicated in several places and within the cavity of this heart is a mass of periblastic material. It would look as though the periblast had been sucked from the surface of the yolk into the heart cavity. Several large periblast nuclei, *pb*, are indicated and are easily recognized on account of their amorphous shape and huge size.

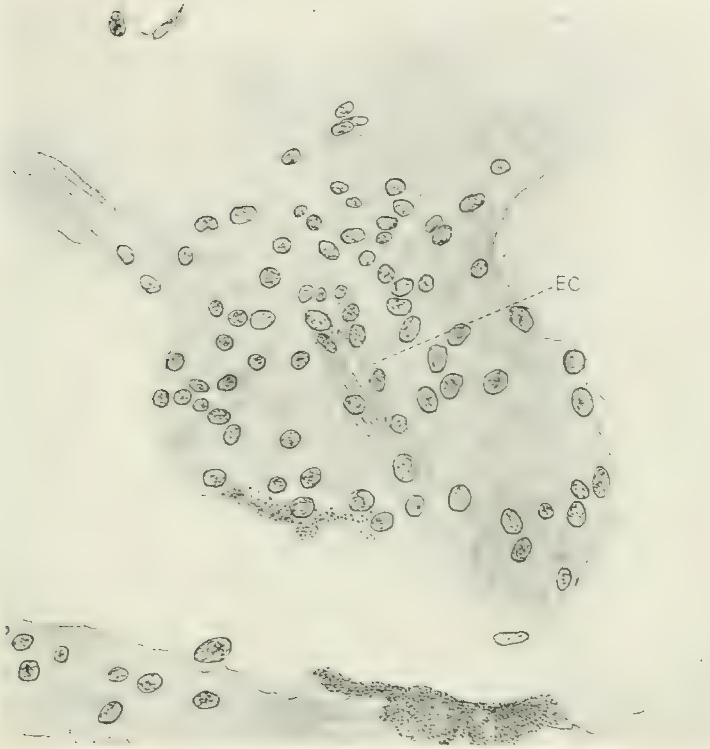
In figures 26 and 28 there are several cells, *c*, of a questionably degenerate type, the cytoplasm of which stains an extremely red color while the nucleus is small and pycnotic in appearance. These cells might in cases be looked upon as some type of wandering cell, but in most instances they are very degenerate in appearance.

It must be distinctly noticed that in none of the figures are erythroblasts shown. Throughout these heart regions at all stages the observer is impressed by the entire absence of any form of red blood cells in embryos that have absolutely had no circulation. One must constantly guard against the possibility of a slight circulation having existed for a short time and then having ceased. Another reason for blood movement may be the twisting or twitching reactions of the embryonic body. Conclusions regarding the permanent position of blood must

Figs. 29 and 30 Two sections through different parts of the heart in an embryo sixteen days old, without a blood circulation; Embryo 314.

Fig. 29 The aortic end of the heart, an almost solid mass with endocardial, EC, cells near the center.

Fig. 30 Through the string-like portion of the same heart; *pb*, periblastic nuclei and material completely fill the heart cavity; *EC*, endocardial cells; *Ch*, chromatophores surrounding the heart wall. The upper part of the section is cut slightly oblique.



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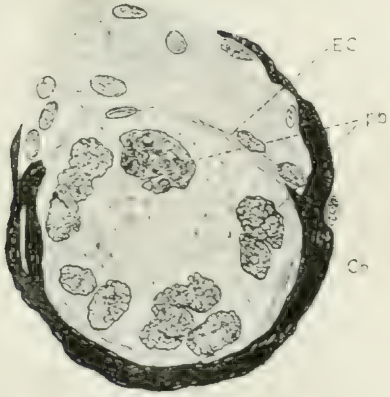

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be based only on embryos that have been carefully observed throughout their existence.

Figures 29 and 30 show sections through different parts of a solid heart string from an embryo of sixteen days old. In figure 29, the aortic end of the heart is shown to be almost a solid mass, and only near the center of the figure is a slight endothelial-like cavity or formation.

Figure 30 is a cross-section through the long string-like portion of this heart. It is seen to be completely solid, the central portion or core consisting of periblastic material containing large amorphous periblast nuclei, *pb.* Chromatophores have almost ensheathed the structure and present in the figure a dense black border. In one part of the section, however, a distinct endothelial-like formation is shown surrounding the periblastic core, and this heart again would seem to have sucked itself full of periblastic material from the surface of the yolk. As stated, this heart was from an embryo sixteen days old in which the blood had never circulated, and it is quite evident that at the time the embryo was fixed, it would have been impossible to have had a circulation of blood through such a solid heart. In this specimen, however, numerous blood islands on the yolk-sac and well formed blood cells in the intermediate cell mass were to be seen.

The endothelial lining of these hearts has certainly arisen *in loco*, and has emphatically not grown into the heart from the yolk-sac vessels since the heart is not connected with such vessels, and further, no typical vessels are present on the anterior portion of the yolk-sac. In all cases, the intra-embryonal vessels are much better developed than the vessels of the yolk-sac. A general survey of these embryos would quickly convince one that the vessels within the embryo are in no case derived from ingrowths. This fact is peculiarly emphasized in a study of bony fish embryos, and is so convincing that it led Sobotta ('02) to develop a theory of vascular outgrowth from intra-embryonic vessels in contrast to the older parablast notion of His ('75), but I must agree with Mollier ('06) in his view that both theories are equally untenable.

The hearts in these experimental embryos as a rule lead directly into a more or less well formed 'aorta which, in all cases shows an endothelial lining. The arches arising from this ventral aorta are very variable in the different embryos, yet in some cases are formed in an almost normal fashion. These arches also show a beautifully formed endothelial lining, but here again one is impressed by the absolute absence of erythroblasts in any stage of development within the neighborhood of the heart or aortic endothelia.

2. The "intermediate cell mass;" its origin, position and significance as an intra-embryonic blood anlage

On tracing the sections posteriorly one finds the intermediate cell mass to begin caudad of the pectoral fins and in the region of the anterior portion of the kidney duct. In studying a progressive series of very young stages forty-eight, sixty-six and seventy-two hours, the intermediate cell mass may readily be demonstrated to arise from the lateral mesodermic plates in the manner so clearly described by Swaen and Brachet ('01, '04). Felix ('97) previously pointed out that the primary lateral mesodermic plates extend away from the somite and later become divided into the following three parts. The median cells lying close to the somites separate away to form a continuous longitudinal string, the string from each side forming one lateral half of the future intermediate cell mass. The intermediate cells of the primary lateral plates just lateral to the above median group give rise to a second cord of cells which later forms the primary nephric duct. The remaining lateral layer of cells now constitutes the secondary lateral plates which split to form two lamellae.

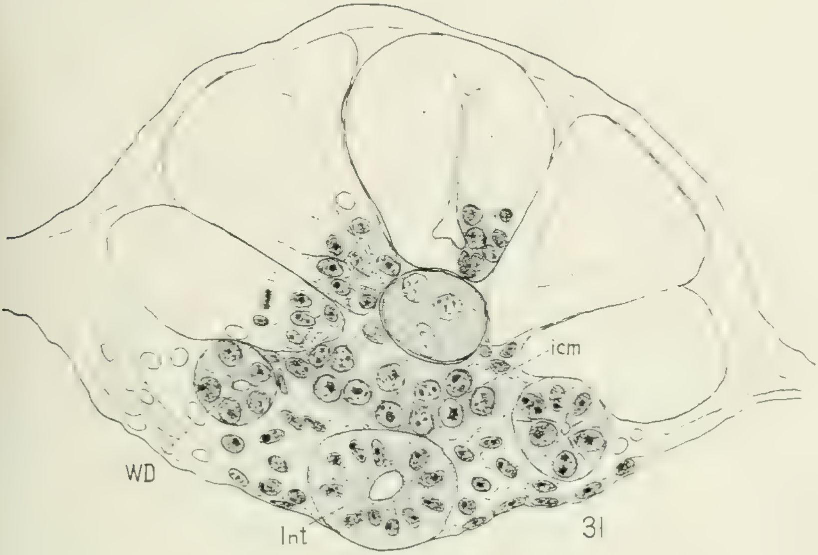
The primary lateral plate mesoderm thus gives rise to the intermediate cell mass, the primary nephric ducts and the somatic and splanchnic mesodermic layers of the lateral body wall. Between these two lateral mesodermic layers arises the portion of the coelomic cavity which we have seen in the living embryos without a circulation of the blood to be greatly distended with fluid.

The later development of the intermediate cell mass is found to proceed in almost exactly the manner described by Swaen and Brachet ('01, '04). This mesenchymal mass of cells is at first of an indefinite type lying between the notochord above and the intestine below and being flanked on either side by the primary nephric ducts. The first notable differentiation of the intermediate mass in the normal embryo begins shortly previous to the establishment of a heart beat. In an experimental embryo of seventy-two hours old, that is one in which the heart was just about ready to begin beating, figures 31 and 32 show the condition of the intermediate cell mass in cross section.

In figure 31, which is the extreme anterior end of the mass and, therefore, less well differentiated than the more posterior regions, the cells are seen to possess large round nuclei differing but slightly from the nuclei of the surrounding cells and those of the epithelium of the Wolffin ducts. The mass of cells is completely unsurrounded by endothelium, and I agree entirely with Swaen and Brachet that the central cells go to form the red blood corpuscles while the cells about the periphery of this mass form the vascular endothelium.

Figure 32 illustrates a section through a more posterior region of the same embryo, the intermediate mass is seen to be much further differentiated. The cells are here typical early erythroblasts and many are observed to be in active mitosis. The cells in the mass are becoming dissociated so that they are no longer so densely packed as in the section through the anterior region. This section is posterior to the ends of the Wolffian ducts, as well as the closed intestine and beneath the cell mass is shown the periblast over the yolk.

On tracing the series still further caudad, we find the indefinite cell mass end Knospe described by Marcus ('05), figure 33. This is a ventral cellular mass into which leads the notochord, intermediate cell mass and end of the endoderm. In other words, this mass would seem to represent the end bud at the dorsal blastopore lip, as if it were the point from which differentiation had taken place or from which the layers had grown forward.



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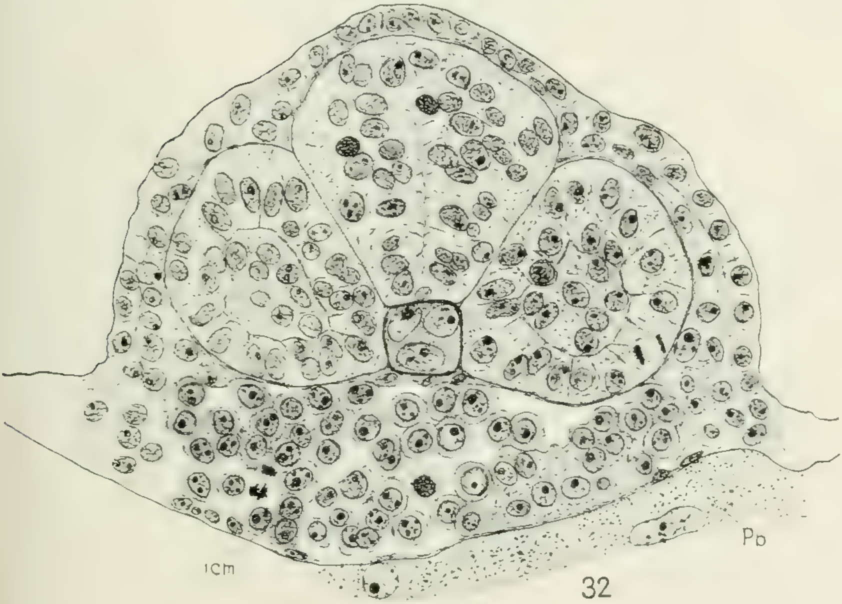

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Fig. 31 Section through the trunk region of a seventy-two hour embryo without a circulation; Experiment A, 1913. The extreme anterior end of the intermediate cell mass, *icm*, is represented by deeper staining cells between the notocord and intestine, *Int;* the primary kidney ducts, *WD*, are lateral to the mass.

Fig. 32 Represents a more posterior section through the same embryo; in this region the intermediate cell mass, *icm*, is more extensive in cross-section and its cells are further differentiated than those in the more anterior region, *Pb*, periblastic material and large nuclei between the embryo and the yolk.

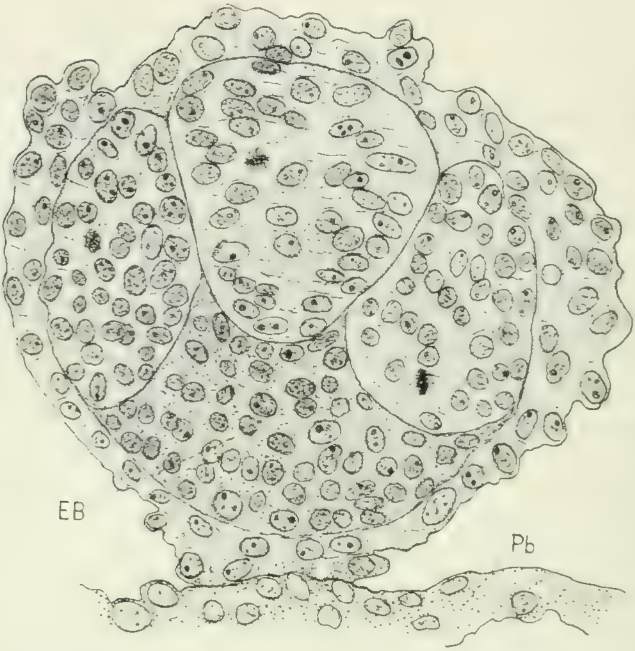


Fig. 33 A still more posterior section through the same seventy-two hour embryo as figures 31 and 32. This section is posterior to the place where the intermediate cell mass and gut endoderm fades out into the indifferent cell mass, *EB*, which may be considered to represent the end-bud mass; *Pb*, the yolk periblast.

The condition in the seventy-two hour embryo is, of course, quite early and the cells are not yet as a rule taken into the circulation. The appearance shown in these figures is exactly that of very slightly younger normal embryos in which a circulation would later be established. The figures, however, were made from sections of an embryo that had no heart beat at the time of its fixation, and, therefore, there is no chance that any of these cells could have become misplaced by having been circulated or carried about.

The most careful study with the highest power of the microscope has failed to reveal any type of cell in the intermediate cell mass other than the early erythroblast, and in later conditions one finds here only red blood corpuscles. In other words, this chief stem blood anlage of the bony fish seems to be a specific red blood cell forming mass. This mass was first discovered by Oellacher in 1873 and has been shown by numerous investigators, Zeigler ('87), Winckebach ('86), Henneguy ('88), Sobotta ('94), Felix ('97), Swaen and Brachet ('99 '01) and others, to be peculiar to the Teleosts.

It is important to know that none of these investigators have yet recorded any type of blood cell arising from this mass other than the erythroblast. Of course, it may be argued that no special study was made of this particular point. Yet it is certainly true that if lymphocytes or leucocytes had been present to any extent, they should have been observed by many of these very capable and careful workers. It has recently been claimed by Maximow ('09), Dantschakoff ('07, '08) and others, that the popular opinion that leucocytes are very late in arising is erroneous since they actually arise just as early as the erythroblast. Then it seems all the more probable that if lymphocytes or leucocytes had been present in this intermediate cell mass such cells would have been discovered, since the mass has been carefully investigated right up to the moment at which it becomes swept into the circulating plasma.

Various investigators have differed as to the vascular products derived from the intermediate cell mass. Some have claimed that it forms only blood cells and no vascular endothelium, Sobotta ('02), while others have attributed the production of cardinal veins or venous endothelium as well as the blood cells to this mass, Felix ('97), and finally others, Swaen and Brachet ('01, '04) in particular, have considered this to be the source of both the aorta and cardinal veins as well as the red blood cells. I have spent considerable time in a study of this question and am inclined to believe that the endothelium of the cardinal veins and aorta arises from the mesenchymal cells surrounding the intermediate cell mass, which are different in nature from the cells actually constituting the mass. Yet it must be admitted that up to the present moment a complete demonstration of the origin of aortic endothelium from the cells about the periphery of the intermediate cell mass has not been satisfactorily shown. This question will be more fully considered in another section.

In the embryos in which the red blood cells remain confined within the median region throughout life these cells develop in a normal manner and become completely differentiated into typical ichthyoid erythrocytes and exist as such for some time. Finally, however, for reasons at present impossible to state but likely associated in some way with an insufficient supply of oxygen, these erythrocytes begin to degenerate and in old embryos of sixteen to twenty days only a very few or in some cases none are left in the large intermediate vessel. Mesenchymal cells seem to wander into the mass of erythrocytes and may take part in their destruction.

Figure 41 is a section through the intermediate cell mass of a sixteen-day old embryo and presents this degenerate condition. The erythrocytes are all small and necrotic and many mesenchymal cells are scattered among them.

As we shall see below, the power of existence of the erythrocytes is very much stronger in the blood islands where aeration is no doubt considerably better than in the intermediate cell mass.

3. Blood islands of the yolk-sac, their origin and development

The question of origin of blood cells on the yolk-sac of the Teleostian embryo has been a much debated topic. Almost all of the earliest workers claimed that blood arose in the yolk-sac islands of the bony fish just as in other meroblastic eggs. The later workers, however, have denied this statement and hold that the bony fish forms an exception to the rule, and is the only type of meroblastic embryo in which blood cells do not occur in islands on the yolk-sac.

It has been frequently admitted by several recent workers that certain wandering mesenchymal cells do migrate to the yolk-sac from the embryo and there form isolated blood cells or small cell groups, but that this blood formation is insignificant in amount as compared with the great blood forming intermediate cell mass.

The yolk-sac of the bony fish is peculiar in this connection. In most meroblastic embryos there is a definite mesodermic layer or membrane between the ectoderm and entoderm of the yolk-sac, and it is in this mesodermal layer that the blood islands arise. When one examines the yolk-sac of the Teleost embryo, the mesodermic layer is found to be largely, if not entirely, absent. Thus, the ectoderm lies directly over the yolk periblast which may be considered to represent the primary entoderm. Between these two layers many long spindle-shaped mesenchymal cells are noticed on careful examination, but these cells in the specimens examined are never arranged in a definite continuous layer.

Goodall ('07) has recently stated that in the sheep embryo, the yolk-sac mesenchyme is not to be considered a continuous layer, but consists merely of diffusely scattered wandering mesenchymal cells. These mesenchymal cells in the sheep as in the fish finally collect into groups and such groups ultimately give rise to the blood islands. In the fish it would seem as though the entire ventral or yolk-sac mesoderm, the chief source of blood formation, had been in its phylogenetic development incorporated or drawn into the body of the embryo as the intermediate cell mass, and only a few cells lag behind or later wander out to form the collections of mesenchymal cells upon the yolk. Ontogenetically there is no longer any indication of a mechanical drawing-in process but the wandering out of cells may be readily observed. It is also easily conceivable that this condition probably differs in different species of Teleosts. Therefore, some species may really form no blood cells in the yolk-sac, while again others might have an almost complete mesenchymal layer in the sac and in such a case would probably give a typical blood island arrangement. Whereas, an intermediate condition would be well represented in the species of Fundulus here studied in which there are numerous disconnected wandering cells later grouping themselves to form the blood islands on the yolk-sac.

The appearance of the wandering cells as they radiate out from the caudal end of the embryo on to the yolk-sac is strikingly similar to that shown by the cells wandering away from the central tissue mass in a living tissue culture. The cells are elongated spindle-form and all are moving straight away from their seat of central origin. This phenomenon is well illustrated by the numerous figures of tissues growing in culture media and I shall give illustrations of it in a special study of this subject now in preparation.

In all of the non-pelagic bony fish eggs investigated up to now, the chief blood forming cells are without exception the intra-embryonic intermediate cell mass, and this mass is claimed to form both vessels and blood. While in the pelagic type of bony fish egg the mass is usually concerned in the formation of vascular endothelium, and the blood cells only arise after the embryo is hatched and free swimming.

This peculiar specialization in intra-embryonic blood formation which seems typical for the bony fish has caused the yolk-sac formation of blood to be almost completely neglected or overlooked by recent investigators. Yet in the species upon which I have experimented there is no doubt whatever that blood islands do arise on the yolk and their origin is from the wandering mesenchymal cells. The wandering cells may be connected in some manner with the intermediate cell mass, yet the presence of the islands cannot be explained in the way Swaen and Brachet ('01) have attempted to account for the yolk-sac blood. They assume that the islands are pushed out laterally as branches or portions of the intermediate cell mass. In many cases no direct continuation of cells is traceable between the yolk islands and the intermediate cell mass and even in extremely young embryos yolk islands may appear on the ventral yolk surface at a great distance away from the intermediate cell mass.

The group of four figures, 36 to 39, indicate the progressive patterns assumed in life by these yolk islands. In the very early stage, figure 36 shows separate collections of cells here indicated by stippling. These groups then become confluent as in figure 37, then more or less net-like in appearance with certain nodes or portions thicker and darker than the general net. In these nodes cell proliferation or blood formation is more active. Finally a typical vascular network arises which goes to make up the capillary yolk circulation of the embryo.

These appearances, as stated above, are not readily distinguishable in the very young embryos, yet with a little experience and a high power microscope any one may convince himself that the blood island formation proceeds to a very definite and considerable degree in these embryos.

Figure 34 represents a cross-section through the yolk-sac of an embryo of seventy-two hours old. The ectoderm of the yolk-sac now becomes two-layered, this continues to thicken as age advances until finally in old embryos the yolk-sac ectoderm is many cells thick and often folded and complex in arrangement sometimes showing villus-like processes. Beneath the ectoderm a group of early erythroblasts or blood cells is illustrated. These cells lie immediately upon the yolk mass here indicated by the heavy dark granules. The appearance of the cells in this blood island anlage are closely similar to those shown in cross sections of the intermediate cell mass in figures 31 and 32. The cell nuclei and general cellular arrangements of the two tissues are seen to correspond in appearance, and the manner of differentiation followed in both cases is identical.

In figure 35, a group of five early erythroblasts are shown which were present in a neighboring blood island. They had loosened themselves from the general island mass and appear very much, if not exactly, similar to the early erythroblast seen separating themselves from the compact mass, the intermediate cell mass (fig. 32). The nuclei in all cases are typically those of early red blood cells and the cytoplasm just begins to stain a very pale pink color characteristic of the halo seen around the young erythroblast. All of the cells shown in these yolk islands, both in the earliest condition of the island and in the late old yolk vessels of embryos without a circulation are invariably of the erythroblast or erythrocyte type. In no case has any type of lymphocyte or leucocyte been present in these yolk islands except as late wandering cells.

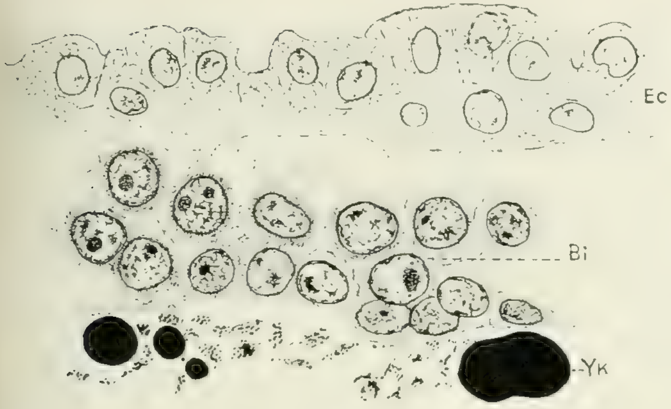
Not all of the wandering cells which are found on the yolk-sac go to form blood cells since many of them are future chromatophores or future endothelial vessel cells. The types, however, are distinguishable in rather early stages and do not seem to be in any way related except that all are of mesenchymal origin. The chromatophores as before mentioned, often come to lie along the walls of the blood vessels.

The early yolk-sac is non-vascular, the blood masses being completely uncovered by endothelium. Later endothelial walls are formed around the blood cell masses and a vascular network is established in the yolk-sac of the normal embryo though poorly formed in the individuals without a circulation. All of these yolk vessels seem to arise by arrangement of wandering mesenchymal cells. Certain of these cells elongate and group themselves in such a way as to form vessel tubes. After the vessels are formed they may then be seen to send off buds and sprouts in the manner Clark ('09) has described in amphibians. The difference between the cells giving rise to the vascular endothelium and those forming the blood cells is not distinguishable in early stages. Yet after considerable study and careful observation, nothing has been observed that would indicate that these vascular endothelial cells possess the power to change into the blood cell type, nor is there any evidence to indicate that cells having once assumed even the earliest blood cell type are capable of metamorphosis to form endothelial cells. It is impossible to state emphatically that the vascular endothelium of the yolk-sac in all Teleosts arises in the same way as that described here for Fundulus embryos. But any one familiar with the very complex yolk circulation of the trout family, in the light of the above knowledge is scarcely justified in assuming that this network of vessels is completely derived from outgrowths from the aorta and cardinal veins within the embryo as Sobotta

Fig. 34 Section through the yolk-sac of an embryo seventy-two hours old, without a blood circulation. A group of cells forming a blood island are distinguished by a slight condensation of cytoplasm about their nuclei: Experiment A, 1913; *Ec*, the ectoderm several cells thick; *Bi*, the cells of the blood island; *Yk*, granular yolk.

Fig. 35 Young erythroblasts just isolating themselves in another island on same yolk as figure 34. Compare the early blood cells with those of figures 31 and 32, in the intermediate cell mass.

Figs. 36 to 39 Illustrate the progressive steps in the development of the network of yolk-sac blood islands.



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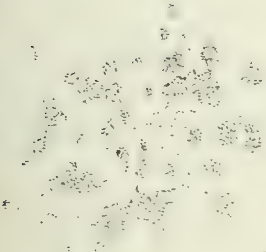

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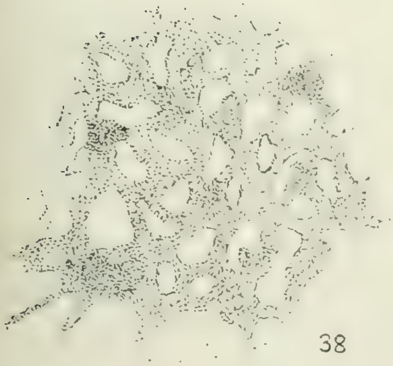

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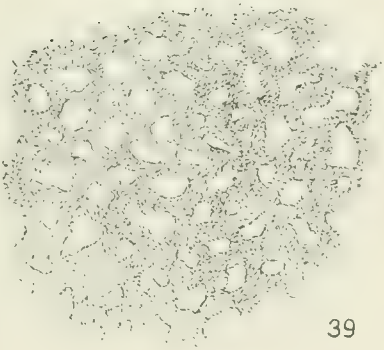

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('02) would have one believe. It is probably true that in the trout family also, wandering mesenchymal cells are of great importance in the formation of extra-embryonic vascular endothelium. There is a strong possibility, as admitted by Swaen and Brachet ('01) that some blood cells are also formed on the yolk-sac of the trout from wandering mesenchymal cells.

The blood cells in the yolk islands increase by mitotic division and soon become very prominent features in those embryos without a circulation, so that in old individuals of eight or ten days the entire posterior and ventral regions of the yolk are almost completely covered with red blood islands. The corpuscles in these blood islands persist in a more or less normal condition for a considerable length of time.

4. Fate of the blood corpuscles in embryos without a circulation

Figure 42 shows the corpuscles in a yolk-vessel of an embryo of sixteen days old in which the blood had never circulated. The vascular endothelium is well formed about the corpuscles and proliferation or multiplication of blood cells has completely ceased, the nuclei are very densely stained and somewhat pycnotic in appearance suggesting a more or less atrophic condition of these erythrocytes.

Figure 40 is a cross-section of a vessel from the yolk-sac of a normal embryo of seven days old. In this the vascular endothelium is also fully developed, large chromatophores have spread themselves along the vessel wall and the erythrocytes are in a vigorous physiological state. The nuclei are lightly staining alveolar structures quite different in appearance from those of the erythrocytes in the older embryo of sixteen days that has never had its blood to circulate. Yet the erythrocytes in the old non-circulating embryo are ladened with haemoglobin and certainly function to some degree.

Figure 41 in the same group is a section through the intermediate cell mass of a sixteen-day embryo without a blood circulation. This is the only intra-embryonic vessel which contains blood cells in this individual. The vascular endothelium is completely disintegrated and has disappeared, and the very degenerate small erythrocytes are now intermixed with mesenchymal cells. In a slightly older embryo, all of these blood cells have disappeared within the tissue as if the invading mesenchymal cells had really assimilated or destroyed the old blood cells.

It is thus seen that in these non-circulating individuals, although red blood cells arise in a perfectly normal fashion and differentiate as completely as in the control embryos, yet they are not capable of maintaining their fully developed condition. Sooner or later they undergo degeneration and finally are completely absent from the body of the embryo.

It is noticed in all cases that very soon after the erythroblasts become completely surrounded by endothelium, they gradually lose their power of multiplication and then differentiate into typical erythrocytes. Before the vascular wall has completely enclosed the erythroblasts, all groups often show many cells in active mitosis, and as I shall bring out below those spaces in which blood cells multiply both in the embryo and in the adult are spaces not completely surrounded by vascular endothelium.

In examining figures 40 and 42, it may be of interest to note that the erythrocytes in figure 40 are the typical ichthyoid type of Minot ('11), while those in figure 42 are what Minot would describe or term, the sauroid type; that is, erythrocytes in which the nucleus has become slightly more degenerate or more densely staining than in the ichthyoid type and in which the cell body is smaller. This sauroid type of corpuscles Minot has designated as being characteristic of reptiles and amphibians, and the condition in these embryos without a circulation indicates the very artificial nature of the proposed classification of Minot. The cells are, of course, ichthyoid but are degenerate and, therefore, assume the 'sauroid type.'

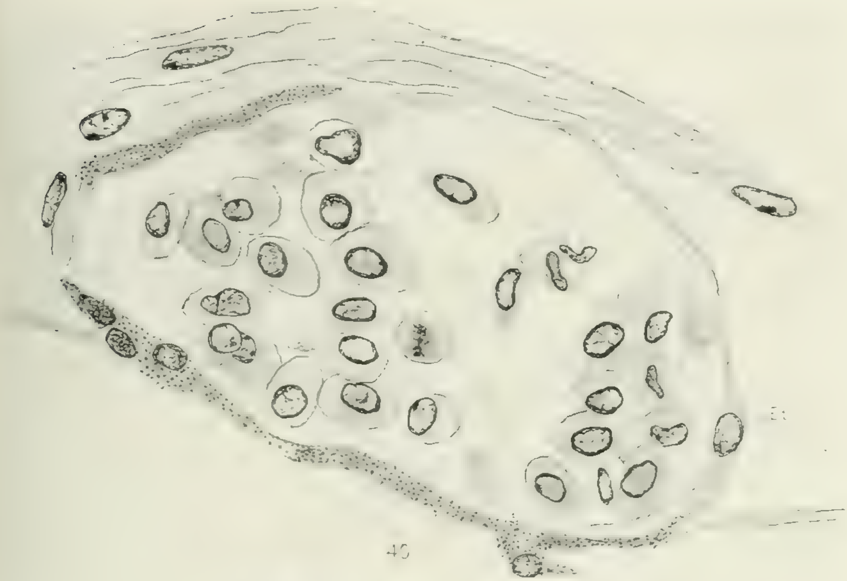
It is difficult for one to believe that all of the functioning erythrocytes in the amphibians and reptiles really have a degenerate nucleus for the simple fact that mammals have blood corpuscles which have completely lost or discarded their nuclei. It must here be remembered that birds are as truly derived from reptiles as are the mammals, in fact, the connection between the reptiles and birds is even closer. Yet the degenerating nuclei of the reptilian blood corpuscle is able to maintain itself in the corpuscles of the bird, although it is according to Minot, so far gone as to degenerate entirely in the corpuscles of the mammal. Such classifications are extremely misleading as they convey to the mind the impression that there is a continuous developmental or evolutionary chain of events illustrated in the blood cells of the different vertebrate groups and actually repeated in the development of the blood in the mammals. The "biogenetic law" is scarcely vigorous enough at present to be submitted to such a strain.

Finally in considering these yolk-sac blood-corpuscles, one must mention the possibility of origin from the yolk periblast or endoderm. It is often stated even in modern text-books and contributions that blood-cells may arise from endoderm and that the primary blood forming layer was actually the endoderm. It is very positively certain that none of the blood cells on the yolk islands of the fish arise from the periblast, but all are derived from wandering mesenchymal cells. The sharp distinction between endoderm and mesoderm is not a thing of any great or definite importance, since everyone recognizes the primary association and origin of mesoderm from the endoderm and the ectoderm. When the mesoderm is once formed, however, it contains within itself a blood forming anlage. It must be further remembered by speculators on the phylogeny of the vertebrate

Fig. 40 A highly magnified section through a yolk-sac vessel in a normal embryo of seven days; *Et*, the vascular endothelium with chromatophores along it. The large beautifully developed erythrocytes are seen in the lumen.

Fig. 41 An equally magnified section through the intermediate cell mass in an embryo without a circulation when sixteen days old; Embryo 413. This is the only intraembryonic blood present, the vascular endothelium, which probably at one time surrounded the erythrocytes, has broken down and mesenchymal cells, *Mes.* are now intermixed with the small degenerate erythrocytes, *Ery*, which should be compared with the normal ones in figure 40.

Fig. 42 Shows erythrocytes in a yolk-sac vessel also in Embryo 413, at the same magnification as in the two preceding figures. These erythrocytes are in a better condition than the intra-embryonic ones, yet they are very degenerate as compared with those of figure 40; all, however, still contain haemoglobin.



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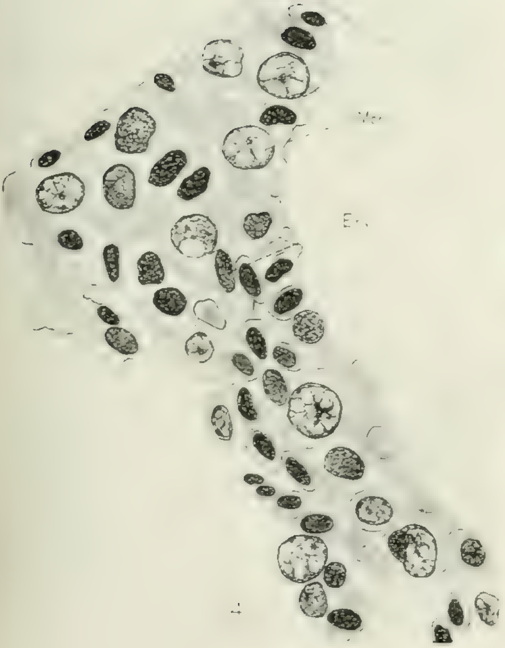

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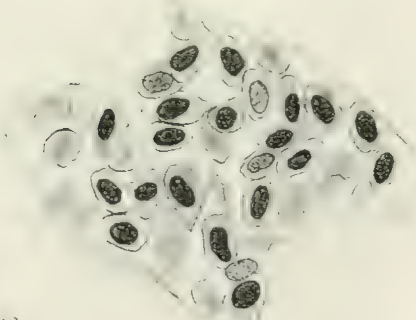

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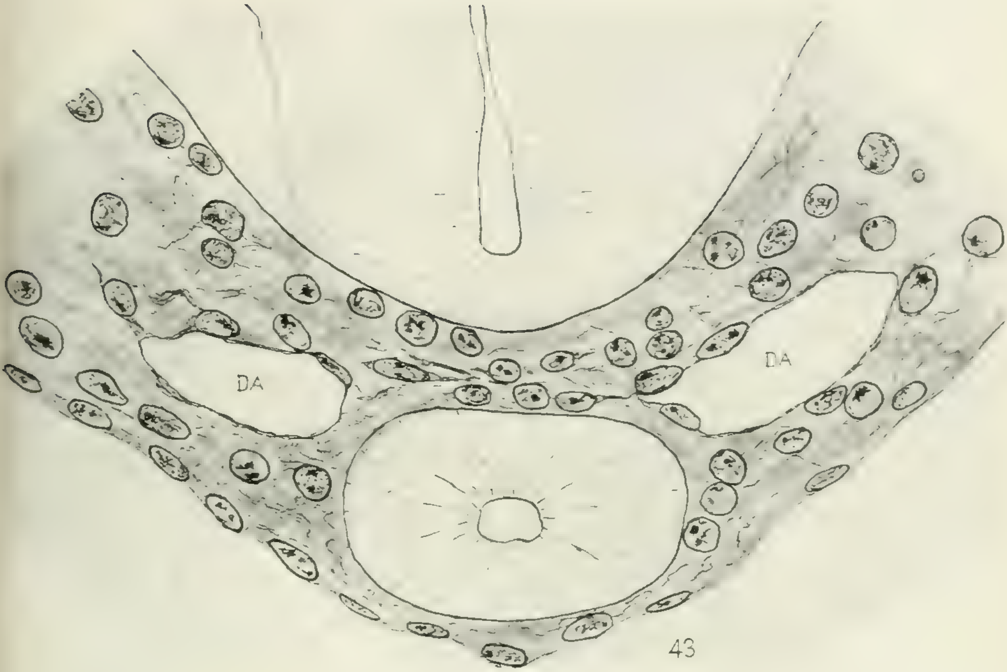
blood that the invertebrate animals, many of which possess highly functionating white blood cells, amoebocytes, as well as oxygen carrying corpuscles, are thought to derive these cells and the vascular endothelium from mesenchyme and not from endoderm.

5. *Has vascular endothelium a haematopoetic power?*

It has been mentioned in describing the origin of blood in various parts of these embryos that no observation could be interpreted to indicate that blood corpuscles ever arise from vascular endothelium. The endothelium of vessels containing blood never presents any cell in a transitional stage. These experiments, I think, furnish a crucial answer to persistent claims that vascular endothelium has the power to change into various types of blood corpuscles. If vascular endothelium had such a power, then one might expect that this power would show itself in cases where it was most needed, for example, in these embryos in which the blood has never circulated. The blood cells are confined entirely to the intermediate cell mass and to the blood islands on the yolk.

The heart and aorta and numerous vessels in the head and anterior portion of the body are lined with typical vascular endothelium, yet in no instance has it been found that one of these vessels contains a single red blood cell in any stage of development. From these experiments, one is warranted in making the bold assertion that the endothelial lining of the heart and aorta is perfectly incapable of giving rise to any type of blood cell. This fact has been mentioned in considering the endothelium of the heart. When we now refer to figure 43, a section through the anterior region of a four-day-old embryo without a circulation, two dorsal aortae are shown. These vessels are lined by typical embryonic endothelium but are completely empty so far as cellular elements are concerned. This is true of the dorsal aortae of all embryos from the earliest to the latest stages when the circulation of the blood has been prevented. Felix ('97) has also noted the fact that the aortae in early normal Teleost embryos are invariably free of blood cells.



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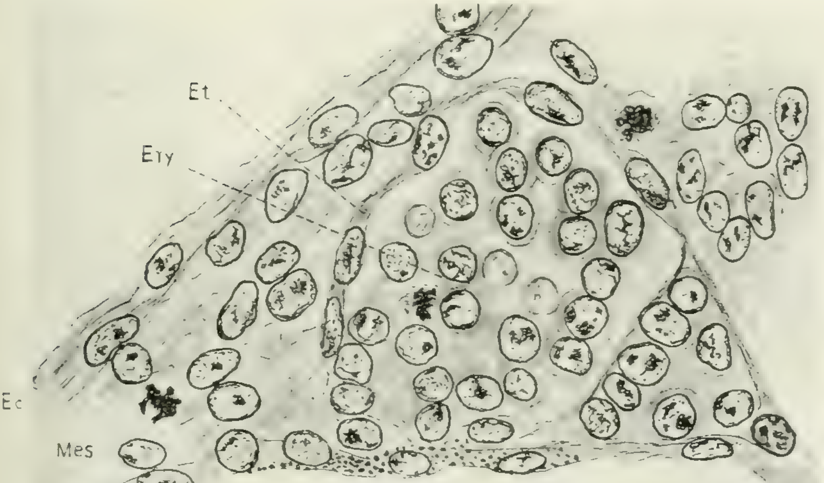

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Fig. 43 Section through an anterior body region of a four-day embryo without a circulation; Experiment 11, 1912, Embryo 6. The dorsal aortae, *DA*, are seen lined by typical embryonic endothelium, yet they are throughout completely empty, never containing blood cells.

Fig. 44 A section through the cardial vein of a similar four-day embryo without a circulation; Experiment 11, 1912, Embryo 4. Again the embryonic endothelium, *Et*, is well formed but the lumen of the vessel is packed with erythroblasts; *Ec*, ectoderm; *Mes*, mesenchyme; *En*, entoderm.

Figure 44 accompanying figure 43 shows a striking contrast in the contents of the cardinal vein. This section is through a more posterior region of the same embryo. The vascular endothelium is here also well differentiated and the vessel is completely packed with early erythroblast some of which are still dividing. None of these erythroblasts, however, have been derived from the vascular endothelium and were actually present before the endothelium was differentiated.

The only source of intra-embryonic blood is from the blood anlage which is contained within the intermediate cell mass, as a rule. But in the splitting away of this mass from between the lateral plate and the somites, it is, of course, conceivable that some future blood forming cells might be left either in the somitic portion or the lateral plate portion. In such cases all those organs arising from regions which had been in contact with the intermediate cell mass either medially or laterally might be contaminated with blood forming cells. If the separation of the blood cell anlage takes place in a clean and complete manner in the individual embryo, then I believe the statement is true that all the intraembryonic blood will be contained in the intermediate cell mass or cardial veins which amount to the same thing.

6. The origin of lymphocytes and leucocytes or so-called white blood corpuscles

We may now turn to a consideration of the origin of lymphocytes and leucocytes or cells other than red blood corpuscles. Many authors have claimed from observations on various embryos that these cells are entirely distinct in their origin from the origin of the red blood corpuscles. Both types, however, arise from the same germ layer or mesenchyme. It has been repeatedly pointed out and seems to be thoroughly substantiated by fact that the lymphocytes and leucocytes in their first appearance are always interstitial in position and are only later contained within the vessels. Whereas, the erythroblasts are invariably formed or divided off into the vessels. In other words, the red blood cell formation tends to be towards or into the vessels and the formation of white blood cells seems to be extra-vascular or interstitial.

It is recently claimed by Goodall ('07) that in the haematopoetic organs of the sheep embryo such as the liver, there are definite groups of proliferating cells forming the various types of white blood corpuscles, and these are distinctly isolated from other groups of proliferating erythroblasts. In the bone marrow this same state of affairs has been described, and in a number of diseased conditions of human marrow I have observed that certain nests or groups of cells were giving rise to leucocytes while other separate groups consisted of erythrocytes. This observational evidence might seem to indicate that white and red blood cells were arising from different parent cells. Yet in normal embryos it is very difficult to obtain material which will conclusively establish such a position, since both types of cells are swept around by the circulation and are intimately inter-mixed in all of the haematopoetic organs.

It would seem that in these experimental specimens in which the blood was prevented from circulating that there might possibly be some way to distinguish completely the source of origin of the white blood cells from the red blood corpuscles if these sources were really different. Should the two types of cells arise from the same common stem cell or parent cell, then the white and red blood cells should be invariably found in association in all embryos. If the two types of cells had different origins they might be found to occur in separate regions of the body and the various sources could thus be readily differentiated.

As frequently stated, in the early intermediate cell mass and among the cells immediately developing out of this mass no leucocytes or lymphocytes are found. The yolk-sac blood islands also consist entirely of cells of the erythroblast type. These observations are in accord with those of all other investigators studying the development of the blood in the bony fish. They have invariably described the intermediate cell mass as being the source of red blood corpuscles and no one has ever recorded either lymphocytes or leucocytes as arising from this mass.

The only cells within the embryo which resemble lymphocytes or leucocytes in their general structure and staining capacities have been found in the anterior portions of the body and in the head region of the young embryos. In very young embryos of seventy-two hours, numerous isolated cells and occasionally small groups of cells are found within the mesenchyme which present a peculiar appearance. The nuclei are more or less dense, the cytoplasm very small in amount in many and in others very extensive, and staining with a color quite different from that of other cells in the embryo.

Figure 45 shows a section through the head just behind the optic stalk of an embryo of seventy-two hours. In this section there is seen a nest of the above-mentioned cells, several are polynuclear and present various peculiar appearances. The mesenchyme within this region is in active mitosis.

Figure 46 is also taken from the anterior end of an embryo and shows two large mesenchymal nuclei with numerous small leucocyte-like cells within the mesenchyme. Numerous pigment granules are also present in these mesenchyme cells. Some of the cells present nuclei of the polymorphonuclear type.

Figure 47 shows an enlarged binuclear cell and indicates the fine granular nature of the cytoplasm. Such cells resemble very closely the embryonic white blood cells.

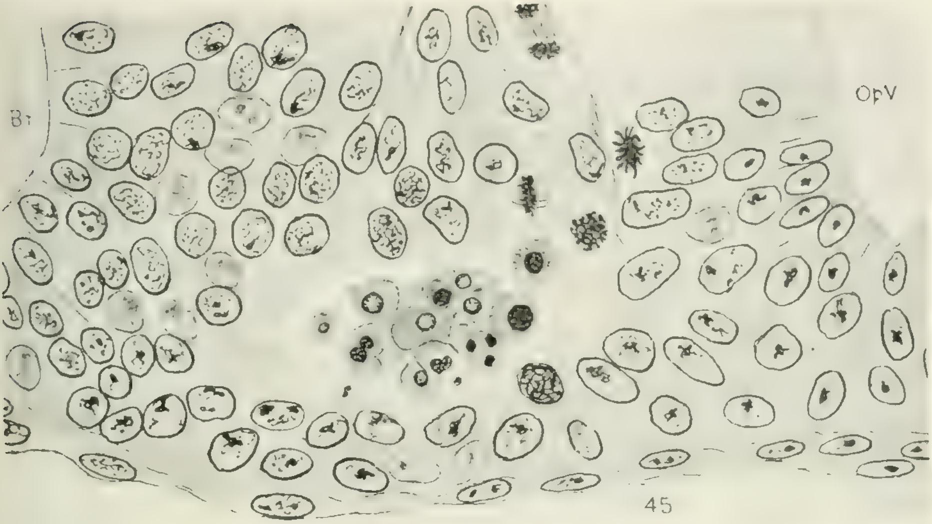
Figure 48 represents a section through the anterior end of an embryo, and shows an endothelial artery which is entirely empty of blood cells. Within the mesenchyme, near the vessel, are two of the leucocyte-like cells. Other cells in these embryos resemble very closely ordinary lymphocytes and these

Fig. 45 A section immediately posterior to the optic stalk in an embryo without a circulation when seventy-two hours old; Experiment A, 1913. A nest of peculiar finely granular cells lies in the mesenchyme which contains many dividing cells; *Br*, brain; *Opv*, optic vesicle.

Fig. 46 Cells from a four-day embryo; Experiment 11, 1912; *Mcn*, mesenchyme nucleus; small leucocyte-like cells are grouped in the neighborhood of chromatophores.

Fig. 47 A binuclear leucocyte from a sixteen-day-old embryo.

Fig. 48 Section through one of the dorsal aortae in a four-day embryo; Experiment 11, Embryo 6; embryonic leucocytes in the mesenchyme.



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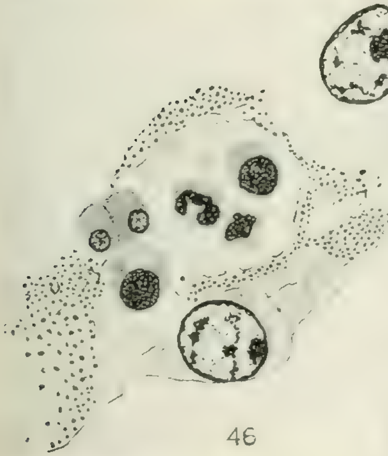

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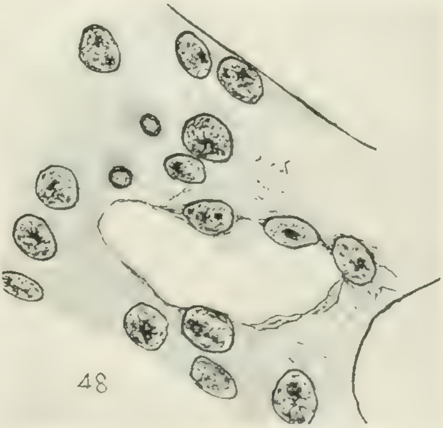

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also are within the tissue spaces and not in the vessels. None of these cells are found in associations with the early red blood cells.

I have examined a number of smears of heart blood, spleen pulp, bone marrow and peritoneal fluid from adult Funduli and have found within these specimens numerous coarse granular leucocytes, lymphocytes and various types of wandering cells. The embryonic cells above described all show a more or less degenerate appearance, but if they can be classed as any type of white blood cell their origin is definitely removed and entirely distinct from that of the red blood cell. In appearance they are as closely similar to embryonic leucocytes as are the cells designated by other investigators to be of that nature. It seems to me that the only possible method of differentiating between the origins of white corpuscles and red blood cells is to prevent their association in the circulating body fluids. These experiments along with numerous observations do show that the red blood cells arise in a region distinctly separated from those localities in which the white blood corpuscles are formed.

When one examines a specimen such as those in which Maximow, Dantschakoff and others have described the origin of white and red cells from a common stem cell, it is impossible to be absolutely certain that the two types of cells do arise from the same individual stem cell. The stem mother cell is shown within the mesenchyme, near this on the one side are various early lymphocytes or leucocyte-like cells, and on the other side are the various stages in erythroblast development. Each type of cell graduates directly back to the stem mother cell or to a mesenchymal cell. This much may be freely admitted, but to say more is merely a matter of guess or interpretation. Since it is absolutely impossible on fixed material to make the definite statement that both of these two types of cells have arisen from the one individual stem mother cell. The observer must actually witness the stem mother cell divide into two cells, and then observe one of these two cells either by differentiation or continued division give rise to white corpuscles, and the other either by differentiation or continued division give rise to erythroblasts before the existence of a common mother cell is proven. This very necessary observation has never been made and all of the evidence in the present literature seems insufficient to warrant the conclusion that such a thing does actually take place, whereas there is considerable evidence to indicate that white and red blood cells probably arise from two different mesenchymal cells. Of course, these two parent mesenchymal cells may be, so far as our powers of observation go, indistinguishable. Yet this would not indicate that they were not different in their potentialities. One of the two mesenchymal cells might be capable of giving rise to the various types of white blood cells depending upon the conditions of differentiation and function, while the other apparently similar mesenchymal cell could on account of its internal difference give rise to erythroblasts. It is a little strange at least that the white blood cells arise far interstitially, while the erythroblasts have such a decided tendency to proliferate into the sinusoids or vascular spaces if they both arise from a common stem cell. The two environments in which they develop could scarcely account for the differences between red and white corpuscles, since in the body of an embryo in which the blood circulates there are several places where the two types of cells develop side by side, as Maximow and others have described. The reasons for the differences are the internal differences between the mesenchymal cells from which the two types of corpuscles arise.

The white blood cells and red blood cells, although both are derived from mesenchyme, arise from mesenchymal cells which have already differentiated sufficienctly far not to be interchangeable. This statement is probably true also of the vascular endothelium in its relationship to blood forming mesenchyme. The embryonic mesenchymal cell if taken in an early enough stage could no doubt give rise to other mesenchymal cells which would later form any of these different type cells. When, however, differentiation has proceeded to some degree, sufficiently far to form what is termed by embryologists an organ anlage, and yet not far enough to make it possible to distinguish between the appearance of various mesenchymal cells, they are then, nevertheless, different in their potentialities.

The mesenchymal cell with the power of forming vascular endothelium is probably very diffusely distributed throughout the embryonic body as well as the yolk-sac. Numerous investigators have supplied evidence indicating this fact. On the other hand, the mesenchymal cells which are to form the erythroblasts are in the bony fish very definitely localized. The latter cells are chiefly confined to the intermediate cell mass, but in addition other erythroblast forming cells wander out probably from the same source of origin, the primary intermediate cell mass, to become distributed on the yolk-sac.

Finally, the mesenchymal cells which are to give rise to lymphocytes and leucocytes of various types seem in Fundulus embryos to be more or less localized in the head and anterior region of the body and do not seem to be particularly associated with vessels. For this reason in early embryos the first apparent lymphocytes and leucocytes are found in the head and anterior body regions, and even in older individuals such cells are more abundant here than in other portions of the body. Yet these cells are doubtless of a roving or wandering type and may finally become scattered throughout the embryo's body. While the non-migrating red blood corpuscles rarely if ever leave their original sites of differentiation.

7. *Environmental conditions necessary for blood cell multiplication and differentiation*

The above facts and interpretations lead us to a consideration of the later conditions of cell multiplication and differentiation. Are the so-called haematopoetic organs of the embryo and even the adult actually haematopoetic, or are they merely favorable localized environments in which various types of blood cells may multiply or reproduce themselves throughout the life of the embryo or individual? There is little doubt, from the recent suggestive studies on the cultivation of tissues in artificial media out of the body of the organism, that certain environmental surroundings are conducive to cell proliferation and growth while other environments inhibit these processes and tend to favor differentiation and functional activity.

Many points mentioned in the previous pages indicate that blood cells change their mode of behavior as the conditions of the embryonic vessels and body are changed during development. A careful consideration of various embryos as well as the different regions of the same embryo suggests that erythroblasts only multiply in spaces unlined by endothelium, whether these be on the yolk-sac, or within the embryonic liver, spleen or bone marrow. The unlined spaces thus afford an environment in which for physical or chemical reasons the erythroblasts are able to multiply and reproduce themselves. When, however, such spaces or channels become converted into endothelial lined tubes then the erythroblasts tend to differentiate into erythrocytes and very soon cease to reproduce themselves any further in this location. In the intermediate cell mass of the fish embryo for instance, one notices in early stages many dividing erythroblasts. Just about the time that this mass becomes completely enclosed by vascular endothelium, this division process slows down and finally ceases although the confined erythrocytes may never be able to leave the vessel. Soon after being surrounded by vascular endothelium the erythrocytes assume a passive non-productive state and remain in this condition throughout their existence.

One of the earliest places of blood cell proliferation or haematopoesis is the sinuses on the yolk-sac. Almost as soon, however, as these sinuses become converted into the yolk vessels, blood cell production ceases in the yolk-sac and only blood circulation takes place.

The haematopoetic processes are then transferred to the embryonic liver and this in most vertebrate embryos is an important seat of blood cell multiplication. The multiplying erythroblasts are never enclosed by endothelium. In other words, they are not within the vessels but their final products are invariably budded or divided off into the sinusoids. Finally, the spaces in which blood cells multiply in the liver become obliterated or converted into endothelial lined vessels and spaces and very soon after this takes place the haematopoetic processes cease in this organ. The liver cells themselves or the interstitial tissues of the liver are not blood forming cells, the only blood formed within the liver arises from existing blood cells which are carried there in the circulating current.

In this manner the multiplication of blood cells is shifted from place to place and becomes more and more localized until ultimately in higher animals the red bone marrow is the only body tissue in which these open spaces furnishing the required environment exist. It is, therefore, the only body tissue in which erythroblasts live and continue to multiply and give rise to the entire stock of red blood corpuscles which circulate throughout the body.

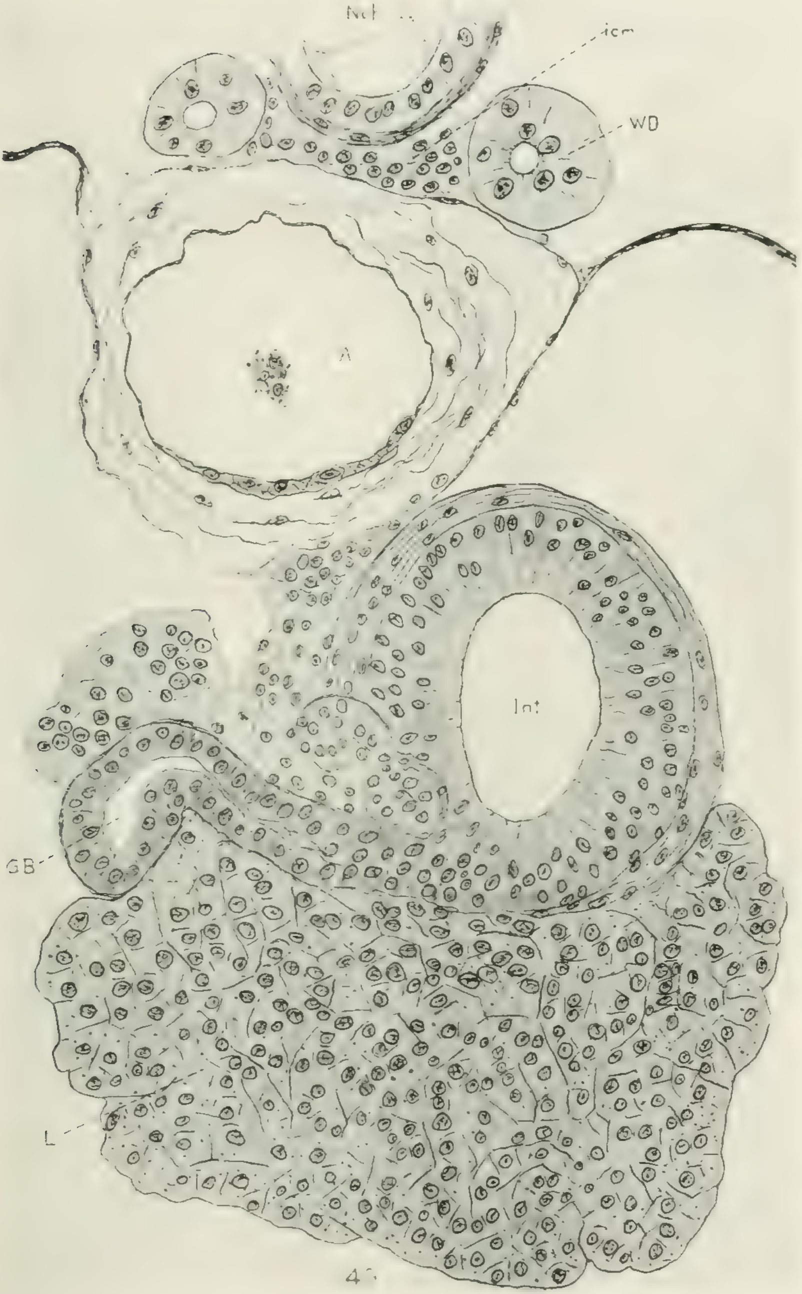
Blood cells always multiply in the unlined spaces but normally never multiply to any extent within closed vessels. It might be possible that certain abnormal growth tendencies on the part of the endothelium of the vessels and sinusoids in the bone marrow might cause an inclusion or vascularization of the spaces in which blood cells multiply and this growth might indirectly result in the cessation of the production of red blood corpuscles. This might be experimentally tested should some method be devised by which the growth of vascular endothelium could be so stimulated as to close the spaces of the bone marrow.

8. Question of haematopoetic organs

In the fish embryo the haematopoetic function of the liver is not of great importance. Yet in the liver of normal individuals numbers of blood cells are always found and numerous dividing blood cells are present. In the non-circulating embryos the blood is unable to reach the liver and in such cases there are no blood cells of any type to be seen in this organ.

Figure 49 represents a section through the gall bladder, bile duct and body of the liver in a sixteen-day old embryo. In

Fig. 49 A section through the liver of a sixteen-day-old embryo, without a circulation; Embryo 413, 1913. The gall-bladder, *GB*, and bile duct are seen connected with the intestine, *Int;* the liver, *L*, is a compact mass containing neither vessels nor any type of blood cell. *A*, the well developed dorsal aorta is lined by endothelium but its lumen is completely empty except for a slight coagulum near the center; *icm*, if followed posteriorly leads into the remains of the intermediate cell mass; *WD*., nephric duct; *Nch*, notocord.



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this individual the heart is a solid string and the blood had never circulated. The liver presents a dense appearance, no blood vessels are seen and blood corpuscles are entirely absent. The general differentiation and condition of the tissues are, however, fairly normal and not at all degenerate. The intestinal epithelium is typical in structure. Above the intestine the well differentiated dorsal aorta is shown with connective tissue fibers abundantly present in its wall and a definite endothelial lining. The lumen of this aorta, however, has never contained any type of blood cells and the only solid particles within it are a slight coagulum near the center of the vessel. Above the dorsal aorta are the two Wolffian ducts and between them under the notochord are a few mesenchymal cells which represent more posteriorly the remains of the intermediate cell mass. Almost all of the erythrocytes in this mass have completely degenerated or have been destroyed by mesenchymal cells.

The embryos without a circulation thus furnish a definite means of establishing the actual haematopoetic value of any organ. They demonstrate that unless the blood current reaches the organ and thereby introduces embryonic blood cells into it the organ iself is incapable of giving rise to blood cells.

This experiment also demonstrates with equal force the inability of vascular endothelium to form blood cells in the fish. I can see no reason if vascular endothelium possesses a blood forming power why the aorta and other interior vessels of these embryos are invariably empty and never contain any type of blood cell. It cannot reasonably be claimed that this inability is due to the abnormal condition of the embryo having taken away the power of the endothelium to form blood cells, since it is so absolutely demonstrated that real blood forming material in other portions of the embryo possesses its perfectly normal capacity to produce blood and does produce it in a very abundant fashion.

These embryos furnish no evidence to indicate that there is any connection or association between the mesenchymal cells which are to form the connective tissue and those destined to form blood cells. There is no instance of a tendency for connective tissue cells to change into blood cells or of blood cells to give rise to any type of connective tissue cells.

Finally, one may conclude that the blood cells like many other specific tissues and organs have a definite localized specific anlage and that this anlage is distinct and separate in most cases from that of the vessel linings. In some cases, however, the blood and endothelial anlagen may come into intimate association, yet even here the two are probably of different mesenchymal origins.

A CONSIDERATION OF THE EXPERIMENTAL STUDY ON THE ORIGIN OF BLOOD IN TELEOSTS IN RELATION TO THE MORE RECENT STUDIES ON THE ORIGIN AND DEVELOPMENT OF VESSELS AND BLOOD CELLS

1. Introduction

This experimental study of the origin and development of blood and vessels relates itself to three more or less separate fields of investigation.

In the first place, the manner in which the blood anlage in Teleosts has separated itself as a unique intermediate cell mass has caused it to be studied as a special subject somewhat isolated from the more general literature on the development of blood in other vertebrates. Yet one very soon appreciates the mistake of this isolation since contributions such as those of Felix ('97) and Swaen and Brachet ('99, '01, '04), in particular, on the Teleosts are of more general importance than most investigations dealing with the broad subject of blood development in the vertebrates. The very fact that in this group the blood anlage is so peculiarly localized in the embryo lends itself as a great aid to the solutions of many questions of haematopoesis or blood genesis.

Secondly, a consideration of the origin and formation of the heart lining or endocardium and the vascular endothelium, in these embryos which have developed without having had plasma or fluids to circulate within their vessels, may furnish much important data towards a final solution of the origin and significance of endothelial lining cells, and the manner of spread and distribution of such cells through the embryonic body and the yolk-sac.

Lastly, such an experimental study bears closely upon the general questions of relationship between different blood cell types. The time and place of origin of the different cells, and the developmental relationship and powers of transmutability existing between various sorts of blood corpuscles as well as the endothelial lining cells of the vessel walls are all problems upon which the experimental results discussed above may throw light.

Each of these three divisions of the problem embraces an extensive and often cumbersome literature which it would be quite out of place to consider in detail at the present time. We shall, therefore, only consider the bearing of the facts recorded in the previous pages upon the opinions and positions maintained by the more recent investigators of the origin and development of blood and endothelium.

2. The specific problems of blood and vessel formation in the bony fish

It may be well to review first the special problems and questions involved in the development of the blood in Teleosts as a group. According to Swaen and Brachet ('01), the mesoblast in the middle and posterior regions of the trout embryo is arranged in two parts, a median primary somite portion and an outer primary lateral plate part. The lateral plate in the mid-body region then divides off a portion immediately adjacent to the somites to constitute the intermediate cell mass. Lateral to this a second part of the lateral plate is separated off to form the primary nephric duct. In the mid-region of the body the somites become separated from the primary lateral plate and the lateral plate pushes or grows towards the median plane and gives off a keel shaped mass between the somites and hypoblast. This mass unites in the median plane with a similar mass from the other side and here forms a large cell group triangular in cross-section, the intermediate cell mass. In the posterior region of the embryo a similar mass pinches away from the primary lateral plate and becomes the posterior continuation of the intermediate cell mass.

Anterior of the first somite in the unsegmented mesoblast of the head this division or pinching away also takes place. Thus the intermediate cell mass of the body becomes continuous with a definite lamella of the head. This well definied topographical portion of the embryonic mesoblast, the intermediate cell mass and cell lamella, is, according to Swaen and Brachet, the only material which gives rise to the heart, the chief vessels and the blood in the embryo. This description by Swaen and Brachet ('01) agrees very closely with that formerly given by Felix ('97), except that Felix disagrees in not deriving the aorta from the intermediate cell mass but from the sclerotoms.

The observations made upon the intermediate cell mass in Fundulus are in close accord with this summary. But no attempt has been made to solve the detailed question as to whether the aorta is derived from the intermediate mass or from the sclerotoms. It would seem that this vessel might arise from either source and still be formed from practically identical cells. Since in the separation of the primary lateral plate from the somite it is easily conceivable that some cells which generally accompany the primary lateral plate might be left as part of the lateral portion of the somite. This lateral portion of the somite is the part which later separates as the sclerotom so that the cells destined to form the aortic endothelium might occur equally well within the intermediate cell mass or within the sclerotom. Their location might vary among different species or even among individuals, and yet these aortic cells would be derived from the same genetic source.

Swaen and Brachet also indicate the head mesoblast as separated into three portions: the intermediate cell mass close to the top of the pharynx, the lateral plate split into two lamellae and the general head mesoblast close around the brain. The intermediate cell mass is more intimately connected with the splanchnic layer of the lateral plate. The pharynx widens in forming the gill pouches which continue to grow dorsally and finally separate the intermediate cell mass into two portions, one part thus comes to lie ventral of the pharynx and the other part dorsal. The ventral portions, at first solid masses below either side of the pharynx, begin to migrate towards the middle line. The two masses fuse into one, spaces are developed in the mass and finally the endothelial lining of the heart is differentiated out of this group of cells. The lamellae of the side plate become separated and the space between them gives rise to the pericardial cavity. Oellacher ('73), Wenckebach ('86), Henneguy ('88), and Sobotta ('94) have all described the origin of the heart in Teleosts in much the same way.

Several of these investigators, Wenckebach, Swaen and Brachet and others, have called attention to a small mass of cells derived from the heart anlage which comes to lie beneath and outside the heart endothelium. This mass of cells has been claimed to wander away from below the pericardium and later to give rise to vessels and blood on the yolk-sac. In the non-circulating Fundulus embryos, however, neither vessels nor blood are formed on the extreme anterior portions of the yolk-sac. I have seen nothing in my studies which would indicate that any cells left over from the heart formation had wandered upon the yolk or given rise to blood cells or vascular endothelium.

Swaen and Brachet are alone in showing that the heart cells are definitely continuous with the intermediate cell mass of the the trunk mesoderm.

Many early workers on the fish embryo have claimed, as has been done for most vertebrae classes, that the heart lining arises from endoderm. The weight of evidence at the present time is so overwhelmingly against such a view that it warrants only a passing mention. Again, however, it must be realized that in the separation of the mesoderm from the endoderm it is possible that some future mesoderm cells may be left behind not cleanly separated. These cells might later isolate themselves from the endoderm to form vessels or blood. It nevertheless seems generally true that all blood forming cells are at one time in development contained within the mesodermal portion of the embryo. Gregory ('02) came to the conclusion that the endoderm and mesoderm could be traced to an indifferent cell mass mesentoderm in certain Teleosts, and according to his view, there is no way to say from which germ layer the heart endothelium actually arises. A mixture of endoderm and mesoderm cells gave rise to endocardium.

The later development of the heart of the bony fish proceeds much as in the case of other vertebrates, as has been carefully described in detail by Senior ('09). The only point of interest in the present discussion is the origin and significance of its endothelial portions, and here Senior after a very thorough investigation confirms in all general points the previous findings of Swaen and Brachet.

In Fundulus as in other Teleosts the heart endothelium partially forms in loco but is also added to by wandering cells or ingrowths of mesenchymal cells adjacently located. The venous end of the heart leads directly down upon the yolk periblast, and as was shown in several figures, this periblastic material with huge amorphous nuclei may be at times drawn up into the cavity of the heart. This would indicate that the venous end was entirely free or not connected with any other vascular endothelium. This condition is, no doubt, due to the absence of the anterior yolk vessels which should in ordinary cases unite or fuse with the end of the heart so as to establish a closed circulation.

According to Swaen and Brachet in the region of the third somite the intermediate cell mass forms only the aorta, while caudad the aorta arises from the dorsal cells of the mass and the great part of the mass forms the red blood corpuscles and the venae cardinales. The endothelium of the cardinal veins finally surrounds the blood cells, but before these cells are fully developed or free, plasma has begun to flow in the aorta and other arteries.

In pelagic forms in which the egg is extremely small and develops very rapidly, the intermediate cell mass in the forward body sections is very small, sometimes only seen between the somites. This portion gives rise to the aorta. The cells are somewhat more numerous in the middle and posterior sections, but they never form a mass to the extent found in the larger demersal eggs. At the time of hatching the posterior cell strings form two lateral longitudinal vessels from the beginning of the mesonephros caudad to the anus. These two vessels, Swaen and Brachet consider to be homologous to the unpaired median stem vein of the trout and this is thought to represent the conjoined cardinal veins. We have noticed that in Fundulus the intermediate cell mass is sometimes divided forming two lateral cardinals loaded with blood cells, while generally it exhibits a median unpaired condition. In the pelagic forms the vessels are all hollow at the time of hatching and the blood cells have not appeared.

Derjugin ('02) claims from a study of the pelagic egg of Lophius that the vessel cells of the aorta and cardinals are derived from the sclerotom. Felix ('97) like Ziegler ('87) differs with Swaen and Brachet ('01, '04) in that he derives the aorta not from the "Venenstrang" but from the sclerotom which under the notochord forms a mesenchymal aortic string. Felix states that no blood cells are to be seen in the aortic anlage, while the cardinal veins, of course, are loaded with the blood cells of the intermediate cell mass. Felix, therefore, derives the two chief vessels of the embryo from two different parts of the mesoderm, the somites or sclerotoms and the lateral plates.

Sobotta ('02) terms the intermediate cell mass "Blutstränge" and derives it from the lateral plate, though he had earlier claimed it to arise from the somites. He described it in the trout embryo in the region from the eighth to the thirty-third somite. The 'Blutstränge' at first paired, are naked cellular strings without a true vessel covering. This they receive later as the cardinal vein anlagen. The endothelial cells of the cardinal veins he derives from the same source which produces the aorta, namely, the sclerotoms.

Finally, then, Swaen and Brachet derive the blood and vascular endothelium of the aorta and venae cardinales from the intermediate cell mass which arises from the lateral plate. Felix derives only the blood and vascular endothelium of the cardinals from the intermediate cell mass which is separated originally from the lateral plate. The aortic endothelium arises from the sclerotoms. Sobotta considers the intermediate cell mass an exclusive blood forming material, while all vascular endothelium, including the heart, is derived from the sclerotoms which are budded off from the somite system. This disagreement, as we have pointed out before, is not of primary importance and may result merely from the fact of the intimate connection of the sclerotom and intermediate cell mass before their original separation.

The question now arises whether all the blood of the Teleost embryo is exclusively derived from the intermediate 'Blutstränge.' Felix admits that the endothelium of the glomerular vessels of the mesonephros arise in loco and at the same time blood corpuscles often occur in this region. Sobotta claims that in the vascular network in the tail of the trout embryo some of the blood corpuscle anlage exists.

Both of these exotic positions of origin may be easily understood. In the first place, the nephric anlage is formed from cells in direct association with those constituting the early intermediate cell mass, and in the separation it probably happens that some future blood cells are held within the kidney anlage and these cells later develop in their proper fashion. The presence of blood corpuscles in the vascular network of the tail is due to the fact that the intermediate cell mass in many Teleosts, as Marcus ('05) has pointed out and as Senior ('09) particularly emphasized extends far back into the caudal region.

A similar consideration is the question of origin of vessels from material other than that of the intermediate cell mass and sclerotom. This is also important, and numerous observations would indicate that in the early bony fish embryo vessels unquestionably arise in loco and not solely as outgrowths or sprouts from a central vessel anlage. Sobotta ('02) on the contrary imagines a gradual growing away of the vascular system from its local origin, the sclerotom. The aorta is the primary vessel and, for example, the sub-intestinal vein arises from the aorta by vascular sprouts which grow around the gut, broaden out and fuse on its ventral side and finally give rise to the longitudinal vein. This theory of Sobotta is as unacceptable in the face of the great body of evidence to the contrary, Felix ('97), Rückert ('88), Hahn ('09) and many others, as is the opposite ingrowth parablast theory of His ('75).

The consideration has been confined so far to the intra-embryonic blood vessels. We may now briefly discuss the development of vessels and blood upon the yolk. There are here two opposed or different views. The first derives the yolk vessels and blood directly from the yolk syncytium or periblast. The second derives blood cells exclusively from the intermediate cell mass in the embryo, but admits that cells may secondarily come to lie on the yolk by being pushed out from the intermediate cell mass with which, however, they maintain a definite continuity. The vascular endothelial cells are also derived from the embryo as mesoblastic wandering cells, but these are not to be compared directly with blood cells since their parent cells have a separate place of origin. Most of the earlier workers thought that the blood in the Teleosts arose on the yolk-sac, as it does in other meroblastic embryos. The more recent workers have gone to the other extreme and deny the presence of blood islands upon the yolk-sac as separate from the intermediate cell mass.

As mentioned in describing the heart formation, numerous investigators have recorded wandering mesenchymal cells upon the yolk-sac, but from a study of the literature no clear conception can be formed as to the origin of blood cells or the vascular endothelial cells upon the yolk from these wandering cells. Some authors claim that the majority of wandering cells become pigment cells, while the remainder form the yolk vessels. In Fundulus the pigment cells very soon present a different appearance from the mesenchymal cells which are to form the vascular endothelium. Both types of cells may be readily seen wandering over the yolk between the ectoderm and periblast. Before the yolk vessels are completely formed, the circulation of a cell free plasma has begun. The extent of the spaces in which this circulation takes place is very variable. The arrangement of the veins of the yolk circulation is also extremely different in the different groups of Teleosts. One must agree with Hochstetter ('93) in stating that the yolk circulation in different forms is so different from the start that it is not possible clearly to summarize the condition in order to give satisfactory comparisons with the same vessels in Selachians, and Amphibians.

When the plasma is flowing in a closed system within the embryo, it is still running as a wandering stream through lacunae and sinuses on the yolk. This probably explains why the blood cells reproduce for so long a time on the yolk-sac while no such reproduction is taking place in the well formed vessels of the embryo.

It is difficult to determine the exact moment, when, or place at which the first blood cells get into the circulation. This probably varies even among embryos of the same species. Ziegler ('88) thinks, however, that just beyond the lateral plates in the plasma filled spaces of the yolk-sac which lie between the periblast and ectoderm, the first blood cells project into the circulation. They are in the form of cell strings which later connect the cardinal veins with the vascular yolk net. Swaen and Brachet saw in trout embryos of eleven days in the region of the fourteenth somite and posterior that the intermediate cell mass spreads out laterally below the lateral plate and on to the yolk surface. The cells thus came to lie above the yolk syncytium and first attained their red color in this position. These authors thus claim that in the bony fish with a large yolk-sac the haemoglobin free early blood cells through continued contact with the yolk become transformed into erythrocytes.

The experimental embryos considered in the present paper demonstrate, however, that it is not at all necessary in such a Teleost to have the erythroblasts reach the yolk-sac in order to acquire their red haemoblobin condition. The tightly packed erythroblasts within the intermediate cell mass of the embryo develop perfectly and readily attain a normal red haemoglobin color.

Finally, comparing the processes of vessel and blood formation in Teleosts with these processes in other vertebrate embryos, we find no definite explanation for the formation of the intermediate cell mass. In other embryos the blood is largely formed upon the yolk. However, it must be recognized from recent contributions that the formation of intra-embryonal blood is much more extensive and important than has formerly been supposed. The relation of the blood anlage to the cardinal vein and the position of the blood forming cells dorsal of the gut are unique in the Teleosts. The late formation of the yolk vessels and their type of origin from wandering mesenchymal cells is also of special interest.

It would seem as though the peripheral mesoblast which in other vertebrate types grows and develops outside the embryo, had in the Teleosts been peculiarly concentrated and drawn into the embryo during its phylogenetic history. Yet in this intra-embryonic position the peripheral mesoblast gives rise to the same cells which it would ordinarily produce on the yolk-sac. The different Teleosts probably show this drawing in of the peripheral mesoderm to various degrees so that in some cases only part of the mesoderm is incorporated in the intermediate cell mass, while the remaining part may still be outspread upon the yolk and there differentiates extra-embryonically. The intermediate cell mass is connected caudally with the end bud, just as the peripheral mesoblast of the Selachians is with the blastopore lip. In its genesis the intermediate cell mass is split off from the lateral plate and localized along its median border.

Marcus ('05) in his study on Gobius capito advanced the opinion that the intermediate cell mass in this embryo is comparable to the peripheral blood forming mesoderm of other meroblastic eggs. In an embryo of eleven somites, the intermediate cell mass passes without a break caudad to the end bud and there connects with both the ectoderm and entoderm, just as the peripheral mesoderm would meet the other two germ layers at the blastopore lip. He attempted to show by diagrams the relationship between the intermediate cell mass in Teleosts and the blood forming mesoderm of Selachians.

As the homologue of the peripheral mesoderm the intermediate cell mass has the power to form vessels and blood cells. Most authors admit this power and only Sobotta ('02) denies the vessel forming property, while others claim that only the cardinal veins arise from the intermediate cell mass, still others, as Swaen and Brachet, would derive the endocardium and aorta also from this common source.

The important fact is that in the small pelagic embryos, where no blood formation takes place before hatching, the intermediate cell mass forms the aorta and the cardinal veins and is also derived from the lateral plate. The lateral plate thus contains cells capable of forming vascular endothelium, and this is the case in all vertebrates.

At an early time in evolution the extra-embryonic blood forming mesoderm has been included within the body of the Teleost embryo and lies over the gut as the intermediate cell mass representing the yolk vascular layer. Here it is important to note that the yolk-sac of the Teleosts contains no real mesodermal layer, only separate wandering mesenchymal cells are found between the ectoderm and periblast, and these wandering cells have migrated out from the embryo.

Finally, as Mollier ('06) states in his review of this subject, it is not a question of the formation of the intermediate cell mass in the individual bony fish, but the wider question of the behavior of the blood forming peripheral mesoderm in the bony fish. All of the results must be considered in this light in their application to other animal classes.

The intra-embryonic blood formation in the bony fish does not represent the primitive type for vertebrates as Sobotta ('02) claims, but this is, no doubt, a modified secondary condition accompanying the various other modified and special developmental processes which bony fish embryo so frequently presents.

Wilson ('91) states of the mesoderm of the Teleost that: "The ventral subvitelline mesoderm, having in this way losts it function in the Teleost, must be regarded as a rudimentary organ of the gastrula. It always remains very small, and does not form any special organ or set of organs in the embryo." The real fact is that the subvitelline mesoderm is misplaced, being within the embryo as the intermediate cell mass and here forms the blood of the individual and, therefore, the yolk-sac of the bony fish has no mesodermic layer.

3. *Vascular endothelium, and vascular growth and development*

Mollier ('06) concludes in his review regarding the origin of vessels as follows.

> As to the genesis of embryonal vessels we may pass the judgment that the theory of the local origin of the vascular endothelium is valuable. The notion of His ('75) and Vialleton ('92) that the vessel strands of the embryo grow in as sprouts from the extra-embryonal anlage (vascular anlage) is not nearly so probable as that the individual vessel cells arise in loco and thus form the vascular nets.

This statement agrees in every way with the contentions so fully presented by Huntington ('10, '14), McClure ('10, '12) and others, regarding the origin of lymph vessels. Lately it receives additional substantiation from the experimental results recorded by Miller and McWhorter ('14) on the origin of blood vessels in the chick embryo. Such a position is further strengthened by the still more recent experimental evidence, presented by Reagan ('15) which shows the origin in loco of vessels in isolated parts of chick embryos. All of these experiments confirm the earlier results of Hahn ('09) on the origin of vessels in the chick.

In the Teleost embryos studied during the present investigation there can be no doubt that the heart endothelium and aortae arise in loco within the embryo, and here there are no vessels, or even mesoderm, present on the yolk-sac in the anterior portion. Certain vessels do partially grow from the embryo out on to the yolk-sac and other smaller vessels arise in many separate regions of the yolk-sac as the products of wandering mesenchyme cells which become arranged to form the tubular vessels. All of these vessels after they have arisen may grow by budding or sprouting off new vessels or may increase in length by a forward growth so well described in living embryos by E. R. Clark ('09, '12) in his careful studies of this subject.

Felix ('97) describes the origin of the aorta as follows:

> The 'mesenchymaortenstrang' arises from the two lines of sclerotoms after they are finally pinched away from the somites. No fusion of cell material occurs between this and the 'venenstrang,' the intermediate cell mass. This 'mesenchymaortenstrang' comes from that

part of the somites that was immediately in contact with the intermediate cell mass portion of the primary seitenplatte. As the forward somites bud off sclerotoms, these also are added to the 'mesenchymaortenstrang.'

The median part of the 'strang' forms the aorta, 'aortenstrang,' the lateral the 'mesenchymgewebe' (mesenchymestrang). *The 'aortenstrang' is at first solid and does not obtain a continuous lumen to begin with, but here and there develops a space, and these spaces become confluent to form the tubes and build the paired aortae.* Certain portions of the strang remain solid much longer than others. The association of the paired aortae to form an unpaired single vessel soon follows. *While the aorta is being so formed, one never finds blood cells within its lumen.* Blood cells occur only in the 'venenstrang' and in certain vessels of the nephric glomeruli. Occasionally certain of the glomerular vessels contain blood corpuscles at a time when the blood circulation is not yet closed.

Felix ('97) cites the observation of P. Mayer ('94) on very young Selachian embryos in which the medulary tube was still open. It was found in such embryos that the aorta is segmental and derived from the somites and subsequently the longitudinal tube is formed by the fusion of these isolated points. Felix agrees with P. Meyer's observations from his study on the Teleost.

There has been great diversity of opinion regarding the germ layer from which the vascular endothelium and blood corpuscles arise. In the literature it may be found that certain competent investigators have in each vertebrate class claimed the vascular endothelium and blood cells to be derived from the endoderm, while other workers of equal authority have found the vessels and corpuscles to arise from the mesoderm. The consistency of the disagreement which one finds in a review of this literature is most peculiar. These disagreements have their foundation in the extreme difficulty of the problem on fixed material.

It is interesting to note that in no case has the same author derived the blood and vessel endothelium from different germ layers. Each author always takes the position that blood and vascular endothelium arise from either the mesoderm or the endoderm.

We have here much to do with wandering cells which become lost from their epithelial layer, and it is impossible to state their origin. This is left to the imagination of the individual investigator and further possibilities of error are open.

Wenckebach's ('86) observations of living embryos are most important in this connection. He noted that not only the layers but that independent mesoblast cells with amoeboid processes wander out of the embryo and over the yolk. These wandering cells play a great part in the formation of the anlage of the heart endothelium and great vessels. In the Teleost embryo one may readily observe these wandering cells in the yolk-sac, and they doubtless give rise to the yolk vessels and blood islands as well as the pigment cells so abundantly present.

Ziegler ('87) has suggested that it may be that the blood anlage in phylogeny has been passed to the mesoderm from the endoderm, and for this reason the endodermal origin may sometimes occur in coenogenetic development. Goette ('90) also held that the endodermal origin of the blood was the more primitive one. This point of view overlooks the fact that in the invertebrates generally the blood and vessel walls are derived from the mesoderm.

In discussing the question of the place of origin of the vessels, Felix ('97) points out that Rückert ('88) claimed in Selachians, that the aorta arose *in loco*. P. Mayer and Strahl ('95), have also stated that the great vessels are late in appearing and arise *in loco* in the embryo's body. Felix states that the glomerulus of the bird mesonephros originates *in loco* independently of the aorta. Further that the stammvene, venenplexus of the mesonephros, certain vessels of the glomerulus, and also the mesenteric artery along with the aorta in the Salmoniden arise *in loco*. Regarding the anlage of the heart and vena sub-intestinales, Felix is not certain but thinks that these likewise arise *in loco*. All of these observations are directly opposed to the theory of ingrowth of vessels from the yolk-sac, the parablast theory of His ('75) as well as the outgrowth of vessels in the sense advocated by Sobotta ('02).

Ziegler ('89) and Felix ('97) have both speculated considerably as to the relationship of the cavity of the circulatory system with the primary body cavity and the coelom. Ziegler pointed out that in the phylogenetic origin of the blood vascular system we have the following changes: The primitive condition is represented by the development of a space between the body wall, the ectoderm, and the gut wall, the endoderm, that is, the primary body cavity or protocoel. Embryologically the blastocoel of the blastular or after gastrulation, the space between the invaginated endoderm and the ectoderm, the schizocoel, represents the primitive vascular space. The body cavity in rotifers, nematodes, bryozoa and arthropods is a primary body cavity and is filled with a fluid, the haemolymph. In the arthropods on the dorsal side of the body is the pulsating heart which sets the fluid in circulation and this fluid contains corpuscles similar to the white blood corpuscles of vertebrates.

In the arthropods the vessels and heart are often highly developed but all communicate with lacunae and spaces between the gut wall and body wall. The heart is surrounded by a pericardial space (not truly coelomic) which is full of haemolymph, and as the heart pulsates this haemolymph is drawn in through ostia along its walls and then propelled out through the aorta and its arches to the vessels and spaces of the body. These body spaces, or the haemocoele, are thought by some to be a secondary or specialized cavity. Yet it is not coelomic and has no definite lining and resembles very closely the primary body cavity of the rotifers, nematodes, and other invertebrate forms which it most probably represents. In some of the higher Crustacea a secondary body cavity or coelomic space of limited extent is present enclosing the ophthalmic artery in Paelamonetes. The cavities surrounding the gonads are also coelomic, and since these are well developed species the coelomic space here probably represents a progressive rather than a regressive condition.

The second step in Ziegler's evolution of blood vessels is illustrated by the conditions in the molluscs. In these animals between the gut and body wall lacunae and interstitial spaces exist which occupy the position of the primary body cavity and these are filled with blood. Vessels lead into the lacunae and the cavities of these vessels as well as the cavity of the well formed heart are also considered to be part of the primary body cavity with which they are continuous. The pericardial cavity in the molluscs is true coelom and not a part of the primary body cavity and contains no blood. In almost all of the molluscs the pericardium is in communication with the nephridia and the nephric duct usually leads from the pericardium to the outer body-wall. The pericardial cavity in contrast to the primary body cavity is designated as secondary body cavity or true coelom.

The final step in the phylogeny of the blood vascular system is characterized by an important expansion of the secondary body cavity or coelom as is the case in the echinoderms, annelids and vertebrates. As a result of the expansion of the secondary body cavity, the primary cavity is reduced merely to a system of channels or vessels and small interstitial lacunae. In the vertebrates, therefore, according to Ziegler, the blood and lymph vascular system represents the persistent part of the primary body cavity. Ziegler considers the blood vascular system and lymph vascular system to have had a common origin. The blood vessel endothelium is closely similar in all respects to the lymphatic endothelium. He thus agrees with Bütschli ('82) that in all metazoa the blood vascular system has its origin from the blastocoel.

Felix ('97) holds that his studies on the Salmoniden will not fit into Ziegler's scheme. He claims that the origin of the stammvene in the cranial portion is the same as that of the primary mesenephros in the caudal region, and is also of the same origin as that of the primary nephric duct. Cells of the splanchnic as well as cells of the parietal layer of the mesoderm enter into the structural material of the stammvene. The cavity of the venenstrang is the same as the cavity between the lamellae of the secondary lateral plate, that is, true coelomic cavity. The three structures referred to are all portions of the same base, the lateral plate mesoderm, the primary seitenplatte. Felix states, as there is little doubt that the cavity of the primary nephric duct is homologous with the secondary body cavity, so there is little doubt that the cavity of the venenstrang is also. The development of the aorta shows similar relations. It arises, according to Felix, from the sclerotomes which come from the somites and contains both the somatic and splanchnic layers of mesoderm. The origin of the aorta from the 'mesenchymaortenstrang' is from the same cell material as the mesodermal layers. The cavity of the myotom is secondary body cavity, coelom, and so also is the aortic cavity. Neither is in any way primary body cavity. The formation of the aortic cavity is a similar process to the canalization of the stammvene. Felix in this way arrives at a conclusion diametrically opposed to Ziegler.

These conclusions he recognizes are not facts but are based on facts obtained from a study of Teleosts which are a side branch of the vertebrate stem, but from which one may still generalize to some extent. Felix calls attention to the fact that in the selachians Zeigler ('92), and in the reptiles Strahl ('83, '85), and in the birds Kölliker ('84) and Ziegler ('92), and in the mammals Kölliker ('84), all claim that the first vessel anlagen are found in the mesoderm and not between the mesoderm and endoderm. Only in the mesoderm the secondary body cavity arises by splitting, and since the solid vascular anlagen are formed within the mesoderm their cavities should not be considered primary body cavity. The writer is entirely unable to agree with such an analysis of the origin of vessels, particularly yolk vessels, as well as of the primary and secondary body cavities for reasons given below.

Felix ('97) now goes further and assumes that the lymph vessels arise in mesenchyme and their cavity is primary body cavity and their wall cells are modified connective tissue cells. This position is difficult to appreciate since it must be admitted that mesenchyme is a direct product of the mesoderm, and, according to Felix, any definitely formed cavity arising between such cells would seem to be coelom. I question, however, whether any other morphologist would put the same interpretation on all the spaces cited by Felix as being in the coelomic category. Felix states, for example, that the aorta arises from a mesenchymaortenstrang derived from the sclerotom. The sclerotom is more or less mesenchymal in nature and certainly contains many cells which will later give rise to types of connective tissue. If the aorta did arise from this group of cells its cavity is scarcely of an origin comparable to that of the coelom. Its endothelial wall is certainly much the same as that of the lymph vessels.

The cavities of the nephric duct, ovarian duct, kidney tubules and other tubules derived from the mesoderm are not usually considered to be parts of the coelomic cavity. The blood and lymph vessels do arise from the mesoderm but not in such a way that their cavity can be readily homologized with the coelomic space originating between the lamellae of the mesoderm. The vessels on the yolk-sac of the Teleosts are formed from disconnected wandering mesenchyme cells which are easily demonstrated. The cavity of these vessels surely cannot be interpreted to arise between mesenchyme cells some of which are derived from the somatic and some from the splanchnic mesodermal layers. The yolk vessels in Teleosts arise by arrangement of mesenchyme cells and so apparently do other vessels within the embryo. Thus these blood vessels are similar in origin to the lymphatics according to Felix's notion of the mesenchymal origin of lymphatics. The numerous recent investigators of the origin of the lymphatics, athough to some extent divided into two schools, all treat the lymph vessels and blood vessels as being of the same general genetic type Sabin ('13) and Huntington ('14).

Finally, the most damaging evidence against Felix's notion that the blood vascular spaces are derived from the coelom, and that these spaces are actually now comparable to the coelomic space is the following: Before a true coelom, such as that to which Felix refers in the vertebrates, has arisen in the animal series blood vessels are already present and these vessels often communicate with or are actually a part of the primary body cavity. When the true coelom does arise in the invertebrate series blood vessels never open into its cavity or communicate with it. Felix has therefore derived an older and more generalized animal system from a newer or later formation. This of course is contrary to any principle of phylogenetic calculations.

The weight of evidence at the present time is then in favor of the earlier notion of Ziegler. The blood vascular system if it is associated with, or phylogenetically derived from any other body cavity, that cavity is really the primary body cavity or embryologically the blastocoel.

4. Haematopoesis, the monophyletic and polyphyletic views, etc.

The experiments recorded above are of particular value in the solution of that very complex problem, the origin and relationship of the different types of blood corpuscles. We may here then briefly consider the evidence they furnish in connection with the various theories and points of view recently advanced in explanation of the origin of blood cells.

The vertebrate animals present two entirely different types of cells floating in their blood fluid. The white blood corpuscles are cells of primitive type and are not only found within the vessels but they also wander through the interstitial spaces of all the tissues of the body. These wandering white blood cells, amoebocytes, are almost universally distributed throughout the animal kingdom being found in all the invertebrate groups above the one or two very lowest as well as in all the vertebrate classes. In no animal do these cells contain haemoglobin, haemocyanin or any compound that would particularly qualify them as oxygen carriers, or give to them any function as an organ of respiration. These white blood cells found outside of the blood currents as well as in the blood are to be looked upon as cells which are not particularly associated with any specific blood function. They merely find the blood current a ready or rapid means of being carried from place to place within the body.

The red blood corpuscles, erythrocytes, are in contrast to the white cells a very highly specialized type of cell and specifically a blood cell. In fact, this is one of the most specialized cells within the body. In mammals, for example, it is specialized to such a degree that its functional perfection is actually accompanied by the loss of its nucleus and necessarily, therefore, the loss of its own future existence after a short period of time.

Contrasted with the almost universal distribution of the white cells within the animal kingdom the erythrocyte is confined to the vertebrates phylum and to certain particular cases among the invertebrates. The respiratory function of the invertebrate blood is often claimed to be confined to the fluid or plasma mass, and only among certain members of the higher groups is a cell developed with the function of carrying oxygen to the body tissues and even this cell can not be said to possess the regular typical characters of the vertebrate erythrocyte.

The vertebrate erythrocyte along with the typical vertebrate mouth, the pharyngeal gills, the dorsal nerve cord, the notochord, and bony skeleton and the many other possessions characterizing the vertebrate group, separates it in gulf-like fashion from the invertebrates. The white blood cells bridge this gulf but the red blood corpuscle differs from that of the invertebrate in a way comparable to the difference between the vertebrate mouth and that of the invertebrate, both serve the same function but are structurally unlike. Just as the mouth and pharyngeal gills and vertebral column have no invertebrate forerunner, so no cell within the invertebrate animals can at the present moment be sought out or designated as the certain ancestor of the red blood cell.

The cells of the vascular walls are closely similar in both vertebrates and invertebrates, as pointed out above. In both animal divisions they probably arise and develop in the same fashion. The white blood corpuscles probably do also. Yet the red cells, although they too originate from the mesenchyme in the vertebrates, are not in any way certainly descended from the invertebrate oxygen carrying cell or the wandering leucoblast or amoebocyte.

There is certainly no phylogenetic or comparative morphological evidence to warrant one in deriving vascular endothelium, leucocytes, and erythrocytes from a common cell ancestry except, of course, they are all derived from the mesenchyme or same germ-layer.

The fundamental histological study of the early developmental stages of the blood elements in vertebrates was contributed by

Van der Stricht ('94). His studies were especially confined to the mammals. As has often been the case the conclusions reached from this pioneer study are largely correct in the light of recent investigations. Van der Stricht held that the first blood cells arising within the area vasculosa are entirely young red cells, erythroblasts. When one surveys the literature of this subject, it is found that all authors with three or four recent exceptions (Bryce ('05), Dantschakoff ('07) and Maximow '09), hold that the blood islands give rise exclusively to red, haemoglobin bearing corpuscles, erythroblasts or finally erythrocytes. This is true for the Fundulus embryos described in this paper and even though the cells are confined to their place of origin and never flow away, since there is no circulation, yet the groups always consistently contain only erythrocytes.

Van der Stricht holds that the leycoblasts and leucocytes are independent of the erythroblasts and arise extra-vascularly in the mesenchyme and later wander into the vessels.

Browning ('05) and Goodall ('07) have both recently claimed that the leucocytes have a different origin from the erythrocytes and arise at a later period. Goodall states:

> When leucocyte proliferation in the liver has begun, the islands of erythroblasts and leucoblasts are definitely separate in position, and the distinctness of their identity is obvious, and no transitions between them can be seen. These facts argue strongly against the view that the erythroblasts are derived from the primitive leucoblasts.

Jolly and Acuna ('05) have pointed out that in early stages only red cells are found in the blood. The first lymphocytes occur very late and still later the granulocytes, so that the guinea-pig embryo has attained a length of sixteen mm. before white blood corpuscles are present.

Again all authors with few exceptions seem entirely agreed that the leucoblasts arise much later than the erythroblast. All without exception also agree that the leucoblasts arise extravascularly while the erythroblasts arise partially within the sinuses, and that the island groups of erythroblasts soon become surrounded by vascular endothelium while no vessel walls have ever been described to form around the groups of leucoblasts. These facts are no doubt of much genetic importance.

The question involved is then: Which is correct, the monophyletic or polyphyletic theory of haematopoesis? It is recognized by all that both propositions are classed only as theory. It must further be recognized that both theories are based at the present time only upon the interpretations of various observers, these interpretations are not necessarily facts. I trust, therefore, that the experiments on Fundulus embryos may add a basis of unquestionable facts which may show the correctness of one or the other of these interpretations.

With this point of view, we may undertake a critical examination of the evidence so ably presented by Maximow ('09) in his study on the mammalian embryo. The observations he construes as strong argument in favor of the monophyletic origin of all types of blood cells and vascular endothelium. This contribution by Maximow ('09) has been accepted by many embryologists *à bras ouverts*, and has been largely incorporated into several recent chapters on the development of the blood, for example, by Schaefer ('12), and Minot ('12).

In the primitive streak stage of the rabbit embryo Maximow states that the peripheral mesoderm in which the blood islands will later occur has in no sense the character of a connected epithelial layer, but consists merely of local accumulations of cells of mesenchymatous type. The cells of this mass are of long thin spindle shape or with star-like processes. These cells are probably much of the same type as the wandering cells seen on the yolk-sac of the Fundulus embryos. In this peripheral mesenchymatous mesoblast the first blood islands arise in the caudal part of the area opaca, as originally described by Van der Stricht. The blood islands are formed from the spindle or branched mesenchyme cells which become associated into groups.

Maximow states that the first endothelial cells like the primary blood cells are also derived from the mesoblast-mesenchyme. With this one may fully agree and several other tissues could be included in the statement as derived from mesenchyme. Maximow, however, goes further and thinks this general source a common specific source. Thus the endothelial cells and blood cells are closely related and arise from a common stem cell in the blood islands and may continue to arise from such a cell during later development.

> Die ersten Endothelien und die ersten Blutzellen sind also beides Mesoblast- resp. Mesenchymzellen. In den Blutinseln sehen wir sie von unseren Augen aus einer gemeinsamen Quelle entstehen. Auch in der späteren Entwicklung werden wir oft Gelegenheit haben, die enge Verwandtschaft dieser beiden Arten von Mesenchymzellen zu beobachten.

This is merely a matter of interpretation and not at all a demonstrated fact. In reply to such a position we must call for an explanation of the demonstrated fact presented on previous pages showing that vascular endothelium forms in a perfectly normal fashion within the heart and head region of embryos without circulating blood, but in no case in early or late stages was the endothelial lining of the aorta or other vessels capable of giving rise to any type of blood corpuscles. Yet the power to form blood corpuscles was abundantly present in the same embryos as shown by the huge numbers of blood cells within the blood forming regions, the intermediate cell mass and yolk islands. Why do not the mesenchyme cells within the liver and all vascular endothelium form blood when no circulating blood reaches them? (If ever, there should then be the stimulus to give rise to its formation).

The red blood cell anlage is a definite mesenchyme cell or group of cells and only members of this cell group possess the blood forming power. To cite a parallel case, the liver cells are derived from the common endodermal cell stock yet not all early endodermal cells, in fact only a few, have the power to develop into a liver, or a pancreas or a lung as the case may be. The embryological argument is indeed rather loose that on account of the fact of vascular endothelium and blood cells arising from mesenchyme would assume, therefore, that these very different cells had a common stem mother cell and later actually possessed some powers of transmutability.

Maximow advances the interpretation that the first blood cells in the area vasculosa are not all erythroblast or future red blood corpuscles. These cells he designates as 'primitive blood cells' since they may form either white or red corpuscles. Yet in the yolk islands of the Fundulus embryos without circulation only red blood cells, erythrocytes, are produced and they remain in this location to be observed throughout embryonic life. The evidence for Maximow's position seems to me somewhat insufficient.

During the summer of 1914, I had the privilege of examining Mme. Dantschakoff's preparations which both she and Maximow cite in support of the monophyletic theory of blood cell origin. One so inclined might interpret these specimens as showing that the red and white corpuscles do arise from the common stem mother cell. The youngest lymphocytes were invariably scattered among the mesenchymal cells while the erythroblasts were budded off into more or less well defined vessels. No one could emphatically state that the two classes of blood corpuscles had ever actually divided off from any one single mother cell.

The more or less constant separation of the early leucoblasts and erythroblasts, as is also shown in Maximow's figures and those of other workers, would seem to indicate their origins from two different mother cells. If one mother cell only forms or divides off cells which develop into lymphocytes or leucocytes and another mother cell gives rise to only erythroblasts, then there is no reason to say that the two mother cells were the same although they appeared to be two similar mesenchymal cells. They were potentially different, and this potential difference is all that the diphyletic notion of blood cell origin demands. A careful study of the embryos without a blood circulation will demonstrate the fact of this different origin of white and red corpuscles.

Maximow then advocates the last clause in the monophyletic code, and states that the intravascular primitive blood cells are not only increased by mitosis but are also added to by the production of the same kind of cells from the fixed endothelial wall of the primitive vessels. Endothelial cells may wander away into the mesenchyme or may wander into the vessel lumen. One often sees according to Maximow a cell project into the vessel, its body assumes a rounded form and its protoplasm changes into that of an erythroblast. It must be distinctly remembered that these appearances are in dead stained specimens and many possibilities exist which might explain their occurrence.

Granting that such a phenomenon actually appears to occur there is one very probable explanation without assuming that true vascular endothelium may form blood corpuscles. Let it be supposed, for example, that in the formation of the vascular wall around the yolk-sac blood islands that some of the peripheral cells of the island might lag behind in their differentiation retaining their more or less mesenchymal type. Such a cell may come to be closely pressed against the vascular wall and really appear as though it were one of the vessel wall cells. This might readily happen, and probably does happen, and may account for the occasional appearance of 'vessel wall cell' forming a blood cell.

Why do not the endothelial cells in the experimental embryos possess the power to form blood cells when the vessel is totally empty of blood cells? Even though it is clearly shown that other cells of the embryo do possess the normal blood building power. These specimens are exactly such as should supply definite proof of blood cells arising from endothelium, but the evidence they furnish really disproves the proposition.

Schridde ('07, '08) according to Maximow has gone so far as to claim that in young human embryos endothelium can directly form primitive erythroblasts. Maximow does not agree with this since in his specimens the endothelium gives rise only to indifferent colorless cells. Shridde's claim is based upon misinterpretation and so, I believe, is any claim that blood cells arise from formed vascular endothelium.

Most authors find that in the very early embryonic blood there are no white corpuscles but only red cells present. Bryce ('05) however, describes in Lepidosiren the very early origin of leucoblasts from primitive blood cells, and later Dantschakoff and Maximow find lymphocytes not only in the vascular net of the area vasculosa but also, though at first very few, in the circulating blood. Maximow thinks that when these early lymphocytes are not seen it is due to poor technique or defective material.

Maximow believes the red blood cells may finally arise from lymphoblasts as erythroblasts, then erythrocytes. This mode of development of the definite erythroblasts continues throughout life and is accomplished in the same manner in all erythropoetic organs. Wherever such indifferent mesenchyme cells, lymphoblasts, are found this locality is eo ipso a new place of origin of erythroblasts out of these colorless stem cells. If this be actually true, why then do not red cells, erythroblasts, finally form all through the body of non-circulating fish embryos, since the wandering lymphocytes surely have the power to reach many places other than the normal sites of erythroblasts formation, the intermediate cell mass and yolk islands?

Maximow claims that both types of blood cells red and white arise at one and the same period from one and the same source, the primitive blood cells in the area vasculosa. The experiments on Teleosts do not bear out such a position since the original or first blood cells from the intermediate cell mass are all erythroblasts and show a characteristic type at a very early time. The blood origin on the area vasculosa is not so extensive, but here also first form only erythroblasts.

Supporters of the polyphyletic origin of blood cells have been able to make equally strong observations in favor of their view on similar material to that studied by Maximow, Dantschakoff and other advocates of the monophyletic theory.

Maximow suggests that since the "primitive blood cell" has no haemoglobin it really stands nearer to the leucocyte than to the erythrocyte, and one might say that the leucocyte arises first in development and the haemoglobin cell later. This is most decidedly not the case in the Teleosts where the primitive mesenchymal blood cell passes directly into the erythroblast without ever showing a stage suggesting either lymphoblast or leucocyte.

With Weidenreich ('05), Maximow takes the position which he had earlier manitained that all non-granular leucocytes and also the wandering cells of the tissues constitute one great cell group. The position determines the direction of their development and only in certain places does one find all types being formed, for example, in the embryonic liver and the adult bone marrow.

In reply to the extreme monophyletic position it may be asked: Why are only erythrocytes present in the old blood islands on the yolk of non-circulating specimens? Why is no cellular blood element present in the aorta and other endothelial lined vessels in the anterior region of similar embryos? Why are wandering "primitive blood cells" unable to form blood in the liver and other positions while blood forming power is present to a vigorous extent in certain regions of the same embryo but from a definite anlage? Wandering cells in the Teleost embryos have to do with yolk-sac blood origin, but these wandering cells are the equivalent of a part of the peripheral mesoderm and always wander out on to comparable regions of the yolk and never wander to other places within the embryo.

Maximow probably criticizes correctly the many artificial distinctions between various leucocytes which are pointed out by some advocates of the polyphyletic theory. My experiments do not bear on this point up to the present time.

Finally Maximow does not imply that a granular leucocyte or red corpuscle can change into anything else, they cannot undifferentiate. He states that before there is any development of granulation or haemoglobin in two different cells, there is really a difference in the cells though we are unable to distinguish it. This invisible difference determines the destiny of the cell to form either a leucocyte or erythrocyte. This is certainly true, but we cannot stop just at this point; these differences must be carried a step further or really back to their actual beginning. Then it is found that although two wandering mesenchyme cells on the yolk-sac of the fish embryo are indistinguishable so far as our powers of observation go, yet they are fundamentally different since one is destined to form only an erythroblast while the other possesses no such power and can form only an endothelial lining cell or pigment cell as the case may be.

This is all the diphyletic or polyphletic school would ask. That is, that certain definite mesenchymal cells are actually the red blood cell anlage and only from these particular mesenchymal cells do red blood cells arise. Here we logically stop, for this is what is conceived by embryologists to be an anlage, back of this we go to certain germ layers and still further back to the developmental potentialities of certain individual cells as followed in studies of cell-lineage and finally we reach the elementary proposition of deriving everything from the original egg cell. But to stop with the tissue anlage we find strong evidence to indicate that certain special mesenchymal cells are designated to form erythroblasts, others leucoblasts, and still others, and these are much more universally scattered throughout the embryo, give rise to vascular endothelium. These latter may really be admitted to form endothelium largely as a response to physical conditions.

SUMMARY AND CONCLUSIONS

The present contribution attempts an experimental analysis of the origin and development of blood cells and the endothelial lining cells of the vascular system. Studies on the origin of blood and endothelium in the normal embryo are rendered peculiarly difficult on account of the important rôle that wandering mesenchyme cells play in this process as well as the perplexing mixture of cells of different origin brought about by the early established circulation. The origin of no other tissue is so confused by mechanical and physical conditions.

The first difficulty has been met by a study of living Fundulus heteroclitus embryos with the high power binocular microscope. The wandering mesenchyme cells may in this way be followed to a great extent. The disadvantages due to the intermixture of cells in the blood current have been overcome by the investigation of embryos in which a circulation of blood is prevented from taking place.

When Fundulus eggs are treated during early developmental stages with weak solutions of alcohol the resulting embryos in many cases never establish a blood circulation. In other respects these embryos may be very nearly normal and the development and differentiation of their tissues and organs often proceeds in the usual manner though at a somewhat slower rate. The heart and chief vessels are formed and the blood cells arise and develop in a vigorous fashion. The heart pulsates rythmically but is unable to propel the body fluid since its venous end does not connect with the yolk vessels and in many cases its lumen is partially or completely oblitered by periblastic material and nuclei which seem to be sucked into the heart cavity from the surface of the yolk.

In these embryos without a circulation of the blood one is enabled to study the complete development of the different types of blood corpuscles in the particular regions in which they originate. There is no contamination of the products of a given region through the introduction of foreign cells normally carried in the blood current.

The *actual* haematopoetic value of the different organs and tissues may be determined in the experimental embryos, and clearly distinguished from the ordinary reproduction or multiplication of blood cells which in the normal embryo would reach these organs through the circulation.

The debated question as to the production of blood cells from vascular endothelial cells may be conclusively answered, at least for the species here studied.

The results and conclusions derived from these experiments may be summarized as follows:

1. The Teleost embryo is capable of living and developing in an almost normal fashion without a circulation of its blood. Red blood cells may be seen to arise and differentiate in these living embryos in two definite localities, one within the posterior body region, and the other the blood islands on the yolk-sac.

The blood cells remain confined to their places of origin, yet they attain a typical red color and may persist in an apparently functional condition on the yolk-sac for as long as sixteen or twenty days. The normal embryo becomes free swimming at from twelve to fifteen days, but these individuals without a circulation never hatch although they may often live for more than thirty days.

All recent investigators have claimed that there are no blood islands present on the Teleostean yolk-sac. Yet the presence of such islands is readily demonstrated in living Fundulus embryos, in normal specimens as well as in those with no circulation.

2. The plasma or fluid in the embryos failing to develop a circulation begins to collect at an early time in the body cavities. The pericardium becomes hugely distended with fluid as well as the lateral coelomic spaces and the Kupffer's vesicle at the posterior end of the embryo. The great distension of the pericardium due to this fluid accumulation pushes the head end of the embryo unusually far away from the surface of the yolk. The heart is thus stretched into a long straight tube or string leading from the ventral surface of the head through the great pericardial cavity to the anterior yolk surface (compare figures 15 to 20).

No blood vessels form on the extreme anterior portion of the yolk-sac, so that the venous end of the heart is never connected with veins, and does not draw fluid into its cavity to be pumped away through the aorta. When the heart cavity does contain fluid it is unable to escape and small floating particles may often be observed rising and falling with the feeble pulsations of the heart.

3. The hearts in embryos without a circulation are lined by a definite endocardium, but the myocardium is poorly developed, sometimes consisting of only a single cell layer. Chromatophores are not present in the wall of the normal heart but in the experimental hearts these large cells ladened with pigment granules are invariably found. The cavity in many of the hearts is almost if not entirely obliterated by the presence of periblastic material and large amorphous periblast nuclei.

The conus end of such hearts leads directly to a more or less closed ventral aorta, portions of the aortic arches are seen in the sections as open spaces, and dorsal aortae are almost invariably seen as typical spaces lined by characteristic embryonic endothelium.

A point of much importance is the fact that *neither these hearts with their endothelial linings nor any portion of the aortae at any stage of development have ever been seen to contain an erythroblast or an erythrocyte.* Cells of this type are completely absent from the anterior region of the embryo.

4. Pigment cells normally occur on the Fundulus yolk-sac and arrange themselves along the vascular net so as to map out the yolk-sac circulation in a striking manner. Loeb has thought that this arrangement along the vessel walls was due to the presence of oxygen carried by the corpuscles within the vessels. In the embryos without a yolk-sac circulation the pigment cells arise but rarely become fully expanded so that the usual long branched processes are represented only by short projections, the chromatophore consequently seems much smaller than usual.

The unexpanded pigment cells, however, wander over the yolk-sac and collect in numbers around the plasma filled spaces. The yolk surface of the pericardium and the periphery of the Kupffer's vesicle are often almost covered with pigment. The hearts are during early stages full of plasma and the pigment cells form a sheath around them, while pigment cells are never present on the normal hearts during the embryonic period.

These facts would seem to indicate that the plasma rather than the erythrocytes contain the substance which attracts the chromatophores and initiates their arrangement along the normal vascular net of the yolk-sac.

5. A definite mass of cells characteristic of the Teleost embryo is located in the posterior half of the body between the notochord and the gut and extends well into the tail region. This so-called 'intermediate cell mass' is the intra-embryonic red blood cell anlage in many of the species.

The peripheral cells of the mass as claimed by Swaen and Brachet or the mesenchyme about the mass, Sobotta, forms a vascular endothelium which encloses the central early erythroblasts.

In individuals without a circulation the erythroblasts arise in a normal manner in this centrally located position, and become erythrocytes filled with haemoglobin. Typical vascular endothelium completely surrounds the erythrocytes which instead of being swept away as usual by the circulating current remain in their place of origin. All of the early blood forming cells of this intermediate mass give rise only to erythroblasts.

6. Contrary to the opinion of most recent observers on blood development in Teleosts, the Fundulus embryos both with and without a circulation possess blood islands on the posterior and ventral portions of the yolk-sac. These blood islands are formed by wandering mesenchymal cells which migrate out from the posterior region of the embryo. They represent all that remains of the peripheral yolk-sac mesoderm in the Teleosts and probably wander way from mesoderm related to that of the intermediate cell mass. The intermediate cell mass may possibly represent the bulk of the peripheral mesoderm which is here included within the embryonic body, while in other meroblastic eggs it is spread out over the yolk. The only mesodermal portion of the yolk-sac in Fundulus is made up of the disconnected wandering mesenchyme cells some of which group themselves to form the blood islands, while others give rise to the yolk vessel endothelium, and still other wandering cells develop into the chromatophores.

7. The non-circulating red-blood corpuscles within the embryo remain in a fully developed condition for eight or ten days and then undergo degeneration. In an old embryo of sixteen days it is sometimes found that very few of the corpuscles in the intermediate mass are still present and these are degenerate. The vascular endothelium has been lost and numerous mesenchymal cells have wandered in and lie among the corpuscles.

On the yolk-sac the corpuscles no doubt have a better oxygen supply and here they maintain their color longer but finally also present a degenerate appearance with small densely staining nuclei and cell bodies much reduced in size.

8. Vascular endothelium arises in loco in many parts of the embryonic body in which blood cell anlagen are not present. This endothelium is in all cases utterly incapable of giving rise to any type of blood cell. This incapacity cannot be attributed to the abnormal condition of the embryo as true blood cell anlagen in the same specimen produce blood corpuscles in abundance

Vascular endothelium in the fish embryo has no haematopoetic function.

9. Neither lymphocytes nor leucocytes have been found to arise in the yolk-sac blood islands nor within the intermediate cell mass.

The embryonic white blood cells are most abundant in the anterior body and head regions, and these cells occupy extravascular positions usually lying among the mesenchymal cells.

The sources of origin of the white and red blood corpuscles in Fundulus embryos are distinct, and these two different types of cells cannot be considered to have a monophyletic origin except in so far as both arise from mesenchymal cells.

The adult blood of Fundulus contains lymphocytes and several varieties of granular leucocytes.

10. There is evidence to indicate that definite environmental conditions are necessary for blood cell proliferation or multiplication. Blood cells do not normally divide when completely enclosed by vascular endothelium. This is the key to the shifting series of so-called haematopoetic organs found during embryonic development.

Erythroblasts lying about spaces unenclosed by vascular endothelium proliferate steadily and give off their products into the spaces from which they find their way into the embryonic vessels. Should such an erythroblast be carried by the circulation to another unlined space it may become arrested there and again undergo a series of divisions giving rise to other erythroblasts. When, however, these spaces become lined by endothelium the blood cell reproduction stops.

It most embryos the earliest blood cell formation occurs in the yolk-sac blood islands. The cells in these islands continue to divide until they become surrounded by endothelium, then the yolk-sac blood islands lose their haematopoetic function and become a vascular net through which the blood circulates. The liver now takes up the rôle of harboring dividing blood cells within its tissue spaces, when these spaces become vascularized by endothelium, here again the blood cells no longer multiply but merely circulate.

Finally, in the mammalian embryo, one organ after another ceases to offer the necessary harbor for dividing blood cells until the red bone marrow is the only tissue presenting the proper relationship of spaces and vessels, and here alone the erythropoetic function exists to supply the red blood cells for the entire body circulation. The red blood corpuscles are always produced so as to be delivered into the vessels and thus very soon occupy an intra-vascular position, while the white blood cells arise and remain for some time among the mesenchymal tissue cells in an extra-vascular position.

11. Lymphocytes and leucocytes along with the invertebrate amoebocytes are all generalized more or less primitive wandering cells, and are almost universally distributed throughout the metazoa.

Erythrocytes are very highly specialized cells with a peculiar oxygen carrying function due to their haemoglobin content. In contrast to the universal distribution of the leucocytes the erythrocytes are only found in the vertebrate phylum, except for a few cases existing in some of the higher invertebrate groups. Yet even in these particular cases the oxygen carrying blood cell never presents the typically uniform appearance of the vertebrate erythrocyte. The oxygen carrying function in invertebrates is usually confined to the liquid plasma.

Typical vascular endothelium is widely distributed in the animal kingdom and appears to be formed from a simple slightly modified mesenchymal cell.

These three very different types of cells all seem to arise from mesoderm—the mesenchyme. Yet the present investigation would indicate that each arises from a distinctly different mesenchymal anlage.

The erythrocyte anlage is localized and perfectly consistent in the quality of its production.

The lymphocyte and leucocyte anlage is more diffusely arranged and not definitely localized in any particular cell group.

The vascular endothelium appears to be formed in loco in almost all parts of the embryonic body, and its formation is absolutely independent of a circulating fluid or the presence of blood cells.

The facts presented seem to indicate that vascular endothelium, erythrocytes and leucocytes although all arise from mesenchyme are really polyphyletic in origin: that is, each has a different mesenchymal anlage. To make the meaning absolutely clear, I consider the origin of the liver and pancreas cells a parallel case both arise from endoderm but each is formed by a distinctly different endodermal anlage, and if one of these two anlagen is destroyed, the other is powerless to replace its product.

LITERATURE CITED

BROWNING, C. H. 1905 Observations on the development of the granular leucocytes in the human fetus. Jour. Path. and Bacter., vol. 10.

BRYCE, T. H. 1905 The histology of the blood of the larva of Lepidosiren paradoxa. Part II. Haematogenesis. Transact. Roy. Soc. Edinburgh, vol. 41, Part II, no. 19.

BÜTSCHLI, O. 1882 Über eine Hypothese bezüglich der Phylogenetischen Herleitung des Blutgefässapparates eines Theil der Metazoön. Morphol. Jahrbuch, Bd. 8.

CLARK, E. R. 1909 Observations on living growing lymphatics in the tail of the frog larva. Anat. Rec., vol. 3.
1912 Further observations on living growing lymphatics: their relation to the mesenchyme cells. Am. Jour. Anat., vol. 13.

CLARK, E. R., and E. L. 1912 Observations on the development of the earliest lymphatics in the region of the posterior lymph heart in living chick embryos. Anat. Rec., vol. 6.

DANTSCHAKOFF, W. 1907 Über das erste Auftreten der Blutelemente im Hühnerembryo. Vorl. Mitt. Folia haematologica, 4. Jahrg., Suppl. no. 2.
1908 Untersuchungen über die Entwicklung des Blutes und Bindegewebes bei den Vögeln. I. Die erste Entstehung der Blutzellen beim Hühnerembryo, etc. Anatomische Hefte, Bd. 37.

DERJUGIN, K. 1902 Ueber einige Stadien in der Entwickelung von Lophius piscatorius. Travaux de la Société Imp. des Naturalistes de St. Petersburg, tom. 33.

FELIX, W 1897 Beiträge zur Entwickelungsgeschichte der Salmoniden. Anat. Hefte, Bd. 8.

GOETTE, A. 1874 Beiträge zur Entwickelungsgeschichte der Wirbeltiere. II. Die Bildung der Keimblätter und des Blutes im Hühnerei. Arch. f. mikr. Anat., Bd. 10.
1890 Abhandlungen zur Entwickelungsgeschichte der Tiere. Hefg 5. Entwickelungsgeschichte des Flubneunauges. Hamburg und Leipzig.

Goodall, A. 1907 Haematogenesis in fetal sheep. Jour. Path. and Bacter., vol. 12, nos. 2 and 3.

Gregory, E. H. 1902 Beiträge zur Entwickelungsgeschichte der Knochenfische. Anat. Hefte, Bd. 20.

Hahn, H. 1909 Experimentelle Studien über die Entstehung des Blutes und der ersten Gefässe beim Hühnchen. Archiv für Entwicklungsmechanik der Organismen, Bd. 27.

Henneguy, L. F. 1888 Recherches sur le développement des poisons osseux. Journ. de l'Anat. et de la Physiol., T. 24.

His, W. 1875 Der Keimwall des Huhnereies und die Entstehung der parablastischen Zeller. Zeitschr. f. Anat. u. Entwickelungsgesch.
1900 Lecithoblast und Angiolbast der Wirbeltiere. Abhandl. d. math. -naturw. Kl. d. K. sächs. Ges. d. Wiss., Bd. 26.

Hochstetter, F. 1893 Entwickelung des Venensystems der Wirtelbiere. Ergebnisse der Anatomie und Entwickelungsgeschichte von Merkel u. Bonnet, Bd. 3.
1905 Ueber die Entwickelung der Dottersackcirkulation bei Scyllium stellare. Arch. f. mikr. Anat., Bd. 66.

Huntington, G. S. 1910 The phylogenetic relations of the lymphatic and blood vascular system in vertebrates, and the genetic principles of the development of the systemic lymphatic vessels in the mammalian embryo, p. 339. Anat. Rec., vol. 4.
1914 The development of the mammalian jugular lymphsac, of the tributary primitive ulnar lymphatic and of the thoracic ducts from the view point of recent investigations of vertebrate lymphatic ontogeny together with a consideration of the genetic relations of lymphatic and haemal vascular channels in the embryos of Amniotes. Am. Jour. Anat., vol. 16.

Jolly, J. 1905 Sur l'évolution des globules roughes dans le sang des embryons des Mammifères. Compt. rend. Soc. Biol., T. 57.

Jolly and Acuna 1905 Les leucocytes du sang chez les embryons des Mammifères. Archives d'Anat. Microscopique, T. 7.

Knower, H. McE. 1907 Effects of early removal of the heart and arrest of the circulation on the development of frog embryos. Anat. Rec., 1.

Kölliker, A. 1884 Die embryonalen Keimblatter und die Gewebe. Zeitschr. fur wiss. Zoologie, Bd. 34.

von Kupffer, C. 1868 Beobachtungen über die Entwickelung der Knochenfische. Arch. f. mikr. Anat., Bd. 4.

Loeb, J. 1912 Heredity in heterogeneous hybrids. Jour. Morph., vol. 23.
1893 Ueber die Entwicklung von Fischembryonen ohne Kreislauf. Pflüger's Archiv., Bd. 54.

Marcus, H. 1905 Ein Beitrag zur Kenntniss der Blutbildung bei Knochenfischen. Arch. f. mikr. Anat., Bd. 66.

MAXIMOW, A. 1909 Untersuchungen über Blut und Bindegewebe. Arch. f. mikr. Anat., Bd. 73.

MAYER, P. 1887 Mitteil, der zoolog. Station Neapel, tom. 7.
1894 Uber die esrten Stadien der Gefasse bei Selachiern. Anat. Anz., Bd. 9.

MCCLURE, C. F. W. 1910 The extra-intimal theory and the development of the mesenteric lymphatics in the domestic cat (Felis domestica). Verhandl. der Anat. Gesellsch.
1912 A few remarks relative to Mr. Kampmeier's paper on the value of the injection method in the study of lymphatic development. Anat. Rec., vol. 6.

MILLER, A. M., and MCWHORTER, J. E. 1914 Experiments on the development of blood vessels in the area pellucida and embryonic body of the chick. Anat. Rec., vol. 8.

MINOT, C. S. 1911 Die Entwicklung des Blutes. Handbuch der Entwicklungsgeschichte des Menschen. Keibel und Mall, Bd. 2, Leipzig.

MOLLIER, S. 1906 Die erste Entwickelung des Herzens, der Gefässe und des Blutes der Teleostier. Handbuch der Vergleich. u. Experimen. Entwick. der Wirbeltiere. Jena.

OELLACHER, J. 1872 Beitrage zur Entwickelungsgeschichte der Knochenfische, I. Zeitschr. fur wissenschaftl. Zoologie, Bd. 22.
1873 Beitrage zur Entwickelung der. Knochenfische II Zeitschr. f. wiss. Zoologie, Bd. 23.

RABL, C. 1887 Uber die Bildung des Herzens bei Amphibien. Morphol. Jahrbuch 13.
1890 Théorie des mesoderms. Morphol. Jahr., Bd. 15.

RAFFAELE, F. 1892 Ricerche sullo sviluppo del sistema vascolare nei Selacei. Mitth. a.d. Zoolog. Station z. Neapel, Bd. 10.

REAGAN, F. P. 1915 Vascularization phenomena in fragments of embryonic bodies completely isolated from yolk-sac blastoderm. Anat. Rec., vol. 9.

RÜCKERT, J. 1887 Ueber die Anlage des mittleren Keimblattes die erste Blutbildung bei Torpedo. Anat. Hefte. Bd. 2.
1888 Ueber die Entstehung der endothelialen Anlagen des Herzens und der ersten Gefässstämme bei Selachierembryonen. Biol. Centralbl Bd. 8, nos. 13, 14.
1903 Ueber die Abstammung der bluthaltigen Gefabanlagen beim Huhn und über die Entstehung des Randsinus beim Huhn und bei Torpedo. Sitzungsber. der K. bayr. Akad. d. Wiss., Bd. 32.

SABIN, F. R. 1913 The origin and development of the lymphatic system. Johns Hopkins Hospital Reports. Monographs, new series, no. 5.

SCHAEFER, E. A. 1912 Textbook of microscopic anatomy. Longmans, Green and Co., New York.

Schmidt, M. B. 1892 Über Blutzellenbildung in Leber und Milz, etc. Zeiglers Beiträge, Bd. 11.

Schridde, 1906 Über Myeloblasten und Lymphoblasten. Verhandig. der Kongresses f. innere Medizin, Bd. 23, Vers., München.
1907–1908 Die Entstehung der ersten embryonalen Blutzellen des Menschen. Verhandl. d. deutsch. path. Gesellsch., 11. Tag. Dresden, 16–19.

Schulte, H. von W. 1914 Early stages of vascologenesis in the cat (Felis domestica) with especial reference to the mesenchymal origin of endothelium. Mem. Wistar Inst. of Anat., no. 3.

Senior, H. D. 1909 The development of the heart in shad (Alosa sapadissima) Am. Jour. Anat., vol. 9.

Sobotta, J. 1894 Ueber Mesoderm-, Herz-, Gefäb- und Blutbildung bei Salmoniden. Verh. d. Anat. Ges. auf d. 8. Vers. zu Strassburg.
1902 Ueber die Entwickelung des Blutes, des Herzens und der grossen Gefässstamme der Salmoniden nebst Mitteilungen uber die Austildung der Herzform. Anat. Heft 63 (Bd. 19, Heft 3.)

Stockard, C. R. 1907 The influence of external factors, chemical and physical, on the development of Fundulus heteroclitus. Jour. Exp. Zool., vol. 4.
1915 An experimental study of the origin of blood and vascular endothelium in the Teleost embryo. Proc. Amer. Ass'n Anat., Anat. Rec., vol. 9.

Strahl, H. 1883 Ueber die Anlage des Gefässsystems in der Keimscheibe von Lacerta agilus. Marb. Sitzungsber.
1885 Der Parablast der Eidechse. Marb. Sitzungaber.
1888 Die Dottersackwand und der Parablast der Eidechse. Zeitschr. f. wiss. Zool., Bd. 45.

Sumner, F. B. 1900 Kupffer's vesicle and its relation to gastrulation and concrescence. Mem. N. Y. Acad. Sci., vol. 11.

Swaen, A., and Brachet, A. 1899 and 1901 Étude sur les premières phases du développement des organs dévisés du mésoblaste chez les poissons Téléostéens. Arch. de Biol., T. 16 and 17.
1904 Étude sur la formation des feuillets et des organs dans le bourgeon terminal et dans la queue des embryons des poissons téléostéens. Arch. de Biol., T. 20.

Van der Stricht, O. 1892 Nouvelles recherches sur la genèse des globules rouges et des globules blancs du sang. Arch. de Biol. T. 12.
1893 Sur l'existence d'îlots cellulaires à la périphérie du blastoderme de poulet. Anat. Anz. T. 8.
1895 De la premiére origine du sang et des capillaires dans l'aire vasculaire du lapin. Comptes rend. de la Soc. de biol. S. 10. T. 2.
1896 Origine des globules sanguins, de l'aorte et de l'endocarde chez les embryons de Sélachiens. Ibidem.

VAN DER STRICHT, O. 1899 L'origine des premières cellules sanguines et des premiers vaisseaux sanguins dans l'aire vasculaire, de chauves-souris. Bull. de l'Acad. Roy. de méd. Belgique. S. 4. T. 13.

VIALLETON, L. 1892 Sur l'origine des germes vasculaires dans l'embryon de poulet. Anat. Anz., Bd. 7.

WEIDENREICH, F. 1904 05 Die roten Blutkorperchen II. Ergebnisse der Anatomie und Entwicklunggeschichte von Merkel u. Bonnet, Bd. 14.
1905 Über die Enstehung der weissen Blutkörperchen im postfetalen Leben. Verh. d. Anat. Gessellch., Bd. 19. Vers., Genf.

WENCKEBACH, K. F. 1885 The development of the blood corpuscles in the embryo of Perca fluviat. Jour. Anat. and Physiol., vol. 19.
1886 Beitrage zur Entwickelungsgeschichte der Knochenfische. Arch. f. mik. Anat., Bd. 28.

WILSON, H. V. 1891 The embryology of the sea-bass (Seranus atrarius). Bull. United States Fish Comm., vol. 9.

ZEIGLER, H. E. 1882 Die Embryonalentwickelung von Salmo salar. Diss. inaug. Freiburg i. B.
1887 Die Entstehung des Blutes dei Knochenfischenbryonem. Arch. f. mik. Anat., 30.
1888 Der Urspring der mesenchymatischen Gewebe bei Selachiern. Arch. f. mikr. Anatomie, Bd. 32.
1889 Die Entstehung des Blutes bei Wirbeltieren. Berichte d. naturforsch Gesselschaft Freiburg i. B. Bd. 4.
1892 Uber die embryonale Anlage des Blutes dei Wirbeltieren. Verhandl. der deutschen Zoolog. Gessellschaft.

ZIEGLER, H. E., and ZIEGLER, E. 1892 Beiträge zur Entwickelungsgeschichte von Torpedo. Arch. f. mik. Anat., Bd. 39.

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A STUDY OF WANDERING MESENCHYMAL CELLS ON THE LIVING YOLK-SAC AND THEIR DEVELOPMENTAL PRODUCTS: CHROMATOPHORES, VASCULAR ENDOTHELIUM AND BLOOD CELLS[1]

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INTRODUCTION

The aim of the present consideration is an analysis of the histogenetic changes passed through by the mesenchymal cells in the living yolk-sac. A study of the origin and development of the blood and vascular endothelium in normal teleost embryos, and in other specimens in which the circulation of the blood had been experimentally prevented, made it evident that a detailed investigation of the development of the living yolk-sac would be most instructive for a comprehensive knowledge of the behavior of mesenchyme in forming the blood cells and vessels. The yolk-sac had been thoroughly investigated in sections and the appearance and location of the earliest blood islands and vascular formations were already familiar.

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[1] The present contribution is a continuation of the author's study of "The origin of blood and vascular endothelium in embryos without a circulation of the blood and in the normal embryo" which appeared in the September number of this journal. This first part is referred to in the following pages as 'the previous paper.'

The egg of the Teleost is particularly adapted to the investigation of such a problem, since its yolk-sac has no definite mesenchymal layer and the freely wandering mesenchyme cells may be clearly seen between the ectoderm and yolk periblast. The remarkable extent to which the cells migrate and the great numbers of such wandering cells impress one with the importance of this cellular movement in embryonic development. The appreciation of this phenomenon also emphasizes the great danger of interpreting developmental processes from a mere study of serial sections. Sections fail to produce a correct impression of what is actually taking place in an area vasculosa. The study of living embryos is absolutely necessary and through it one quickly becomes acquainted with the remarkable rôle played by wandering cells in the formation of the heart and vessels, as well as the production of the future blood cells.

These facts have been pointed out long ago, but with little effect, as is indicated by the enormous literature containing the endless interpretations and guesses of numerous authors after studies of fixed and sectioned material. It is not intended to under rate the importance of the study of sections. However, such a study to be fully comprehended must be accompanied by observations on the living material as far as is practical. These observations are further greatly clarified by an experimental modification of the normal developmental processes where such is possible.

Almost thirty years ago Wenckebach ('86), at that time a young medical student in Holland, described his observations on the living embryos of the developing bony-fish. In this contribution he lamented the fact that the knowledge of the embryology of the bony-fish, as well as of other vertebrates, was based almost entirely on studies of sections of the embryos. The germ layers were described actually as growing layers and from this layer formation the different organs were built or produced by foldings. Each cell was thought of as being passive and only through its division was the formation of the organs brought about. This initial investigation, and the only one so far as I know, by young Wenckebach gave him an entirely different view point regarding the processes of embryonic development.

Wenckebach readily observed the cells of the mesoblast independently wandering in amoeboid fashion, often with extraordinarily long protoplasmic processes, within the body of the embryo as well as upon the hypoblast-free yolk. The wandering cells move as with aim and purpose to form certain definite organs. In the formation of the anlage and further development of the heart, as well as the vessels and other structures, this independent wandering of the mesoblast cells performs a most important part.

Unfortunately, Wenckebach's scientific efforts ceased and his early study was unappreciated since on only one occasion was it considered by an investigator, Raffaele ('92), who studied similar material. Today the student gathers from text- and hand-books as well as descriptive embryological contributions much the same orthodox conception of the layers and foldings as all important factors in the origin and development of embryonic organs. No doubt the growth of layers and folds does contribute its part, but this part is almost negligible in a study of the development of the vascular system and blood.

Wenckebach and Raffaele, in their observations of the wandering cells on the yolk-sac failed to recognize the erythroblast. They were also unable to follow the processes in the differentiation of pigment and endothelial cells in the way at present possible with improved microscopes. The experimental embryos without a circulation of the blood are also most instructive for comparison in such a research.

The wandering mesenchymal cells on the yolk-sac differentiate into four distinct types of cells. The chromatophores of two varieties, one black and one of a reddish brown color, endothelial lining cells of the yolk vessels and islands of erythroblasts may all be seen to form in the living specimens. The association of the four types is also clearly determined. In addition to these cells the large periblast nuclei are conspicuously seen. The periblast never gives rise to any type of tissue or cells, but finally the nuclei become swollen and distorted and degenerate in the manner Wenckebach long ago described.

MATERIAL AND METHODS OF STUDY

The material used for these observations and experiments was the embryo of the Teleost, Fundulus heteroclitus. The eggs of this fish are very transparent and may be readily observed by transmitted light with a high power microscope. A compound binocular microscope made by E. Leitz has been found to serve splendidly for such observations since with this instrument an oil immersion lens may be used to study the embryo suspended in a hanging drop from a thin cover glass over a hollow slide. A double or triple lens condenser facilitates the regulation of light and in a darkened field the almost transparent cells may be seen while granular or pigmented cells are distinctly outlined.

The mesenchyme cells are of sufficient size to be readily followed with a Zeiss DD objective while the eggs are grouped on the bottom of a watch glass. An ordinary microscope serves almost equally well for these observations but the Leitz binocular has the advantage of producing an apparently stereoscopic effect, since it permits the observer to look with both eyes at the same time. One is also enabled to see much better and look much more continuously than with one eye. An ordinary binocular microscope is unfit for the finer observations on account of the poor arrangements for condensing the light, and the magnification is insufficient for details of structure.

Observations have been made on the normal embryo at all developmental stages. Specimens in which the circulation of the blood was never established were also used, since in these the behavior and development of the cells of the yolk-sac are in no way contaminated by the introduction of additional cells brought in the circulating current. Such specimens enable one to hold the yolk-sac in its original condition so far as cellular elements go, and further give an opportunity to test the influence of the circulation on the mode of differentiation and function of the mesenchymal cells.

The prevention of the circulation has been accomplished in the same manner as employed in the previous investigation and fully described in the September number of this journal. The eggs shortly after being fertilized are placed in solutions of alcohol in sea-water. The series of solutions most advantageously used is prepared as follows: 1.5 cc., 2 cc., 2.2 cc., 2.4 cc., 2.6.cc., 2.8 cc., and 3 cc. of 95 per cent alcohol is added to 50 cc. of sea-water. These solutions are renewed after twenty-four hours and after another twenty-four hours the eggs are placed in pure sea-water.

Such a series gives, of course, gradiations of the effect. Eggs in the weaker solutions develop normally in many cases, while other individuals develop slightly slower than the normal and have the circulation of their blood arrested to different extents. Many individuals in all of the solutions fail entirely to establish a blood circulation and although the heart pulsates feebly it does not propel the plasma for one or another reason which has been previously discussed. In spite of the failure of the blood to circulate, the development of the cells on the yolk-sac progresses in an almost normal fashion and vessels and blood corpuscles arise in this region and may be carefully observed throughout the life of the embryo.

The observations on the living yolk-sac have been supplemented by a study of fixed and cleared specimens. Embryos at different stages of development are fixed in a saturated solution of corrosive sublimate to which 5 per cent of glacial acetic has been added. Eggs are left in this mixture for 4 or 5 minutes, then rinsed in tap water and placed in 10 per cent formalin. The formalin is changed after about one-half hour when it has become slightly cloudy. This method if carefully handled brings out in a most beautiful way the cell outlines of the ectoderm of the yolk-sac (fig. 34). The yolk remains rather transparent and the mesenchymal cells may be observed beneath the clear cut net-work formed by the ectodermal cell borders. This method has been frequently employed by other workers and I have used it myself for ten years on Fundulus eggs, but have never before succeeded in getting this heavy outline of the cells. It seems scarcely possible that so striking an appearance could have been overlooked, yet it is perfectly simple to obtain. Fundulus yolk-sacs fixed in this way are equally as beautiful as silver preparations of cell boundaries.

In addition to the above, I have used for the first time another solution which renders the specimen still more transparent. This is a mixture of strong formalin 5 parts, glacial acetic 4 parts, glycerine 6 parts, and distilled water 85 parts. Eggs are placed directly into this and left for two days, and then transferred to 10 per cent formalin for permanent preservation. The fluid mixture causes the egg to swell to some extent but it leaves the yolk as clear as in life and by fixing the cells causes them to stand out in beautiful contrast (figs. 5, 6, etc.). The mixture of glycerine and glacial acetic has been used for a long time in preparing transparent specimens of invertebrate eggs. Wilson in 1892 used it with Nereis eggs. The proportions here employed have been used by several students at Woods Hole and are not original with me, except that others leave the eggs permanently in the mixture while it seems better to put them in formalin after two days. The eggs remain equally transparent in formalin.

The cleared specimens are most valuable for use in connection with the studies of the living. But the remarkably beautiful filamentous processes of the wandering mesenchyme cells and the endothelial lining cells of early vessels are not so extensive as during life. Some shrinkage or contraction of these processes always accompanies fixation. The movement of the processes in life also gives one a much better conception of their form and structure.

THE EARLY WANDERING CELLS

About two hours after fertilization the eggs of Fundulus heteroclitus have undergone the first division and are in the two-cell stage. The cleavages then continue in a more or less regular fashion to form a discoidal mass of cells as a cap on the yolk. At eighteen to twenty hours the germinal disc is beginning to flatten or thin out in order to begin its expansion to cover the yolk sphere. After the fourth or fifth cleavage some of the peripheral cells of the germ disc are somewhat fused with the yolk mass and do not present a clearly formed distal cell wall. The nuclei of such cells continue to divide and begin to wander or are pushed out into the superficial yolk material. In this way are formed the so-called periblast nuclei, or more correctly periblast syncytium, of the teleost. This periblast syncytium precedes the germ disc in its descent over the yolk, so that one observes loosely scattered nuclei of unusually large size forming an advance border around the periphery of the germinal disc. The nuclei multiply and finally lie scattered over the entire yolk surface by the time the germ ring or blastodisc has completely covered the yolk (figs. 5, 7 and 8).

These periblast nuclei are of interest to us in the present consideration only on account of the fact that they are located in a superficial syncytium covering the yolk. It is over this syncytium that the mesenchymal cells wander. The periblast of the hypoblast-free yolk-sac of the teleost, in so far as position is concerned, may be compared to the endodermal covering of the yolk-sac in other meroblastic eggs.

The outer cover of the yolk-sac in Fundulus is formed by the germinal disc as it grows over the yolk. This constitutes the ectoderm of the sac which is its only true or typical layer. Thus the yolk-sac consists of an outer-continuous ectodermal layer beneath which are freely wandering mesenchymal cells and below these the periblastic syncytium fuses into the yolk material itself.

The periblast nuclei were interpreted by Agassiz to represent the survival of the nuclei which had at one time in phylogeny controlled the segmentation of the yolk. These were the nuclei of the former yolk laden cells in the holoblastic cleavage of the ancestral teleost. Others have thought that they played some part in the formation of the ventral wall of the gut, etc. In Fundulus, however, they take no part in the formation of the body tissues or organs, but may be observed to degenerate in the late embryo. The periblast nuclei become very much vacuolated, irregular in shape and huge in size before their final degeneration. After the embryo has hatched the remains of the yolk contain a protoplasmic mass in which the periblast nuclei are packed together and the whole is finally absorbed.

The blastodisc is separated from the yolk by a space which arises during the early hours of development. This space between the ectoderm and periblast has been interpreted by Agassiz and Whitman ('84), Ryder ('87), Wilson ('90) and others to represent the blastocoel or segmentation cavity. It is actually into this space that the wandering mesenchymal cells migrate and we shall later recall this fact as of importance in interpreting the nature of the vascular lumen in connection with other body cavities or spaces.

Twenty-four hours after fertilization the germinal disc is from one-quarter to one-third way over the yolk-sphere. Its wall has thinned out centrally and around the periphery is seen a thickened border, the so-called germinal ring. After about 48 hours the germ-ring has traveled almost completely over the yolk-sphere and now surrounds the small remaining uncovered pole of the yolk which may be considered a yolk-plug. A shield-shaped thickening, beginning about the twenty-fourth hour, extends from one region of the germ-ring towards the animal pole. This is the embryonic shield and along its median line a second thickening begins to appear which is the first indication of the embryonic body.

From the edges of the embryonic shield and from the germ-ring as it finally encloses the yolk, an early migration of mesodermal cells takes place. The cells apparently do not wander far and some of them may again be included in the embryonic body. After the germ-ring has enclosed the yolk, from 45 to 50 hours usually, a very active migration of mesenchymal cells begins from the caudal and posterior lateral portions of the embryo. Figure 1 shows outlines of a few such cells wandering out from the side of an embryo of 45 hours. This figure is a camera sketch from life and even at this early time there are some cells inclined to be more or less spindle or stellate in shape with long delicate filamentous projections, while other cells are of more irregular shape with short amoeboid processes. Figures 3 and 4 show the cells highly magnified and in active movement; figure 3 the spindle-shaped delicate process type and figure 4 the heavier more amoeboid cell. These cells are actively wandering and changing in shape as figure 4 shows. From *A* to *E* are the outlines presented by a single cell at five minute intervals.

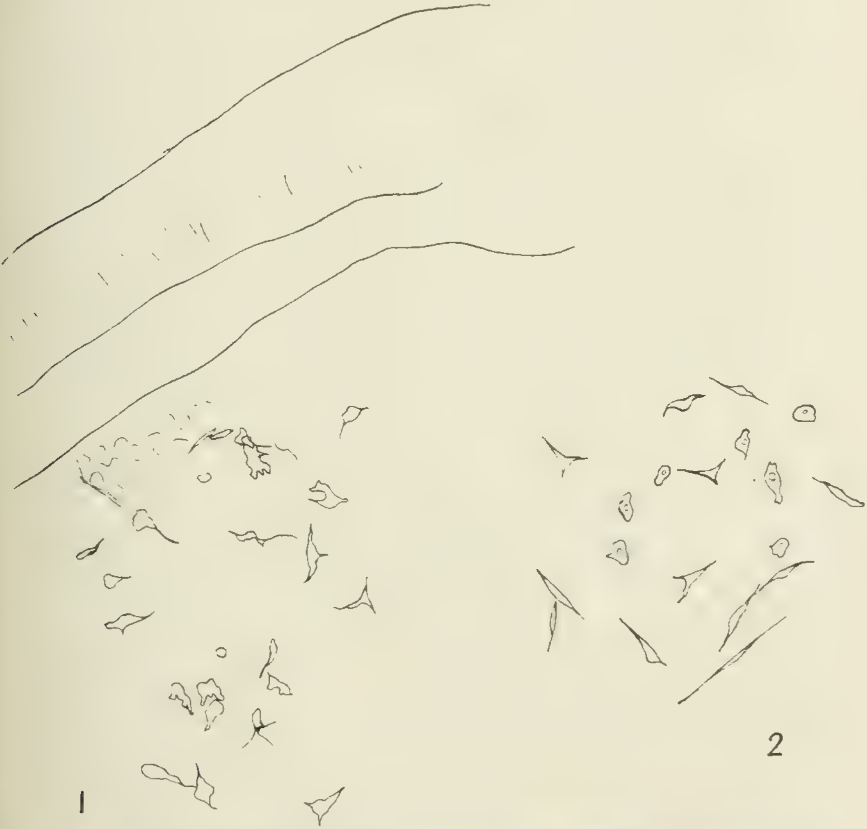
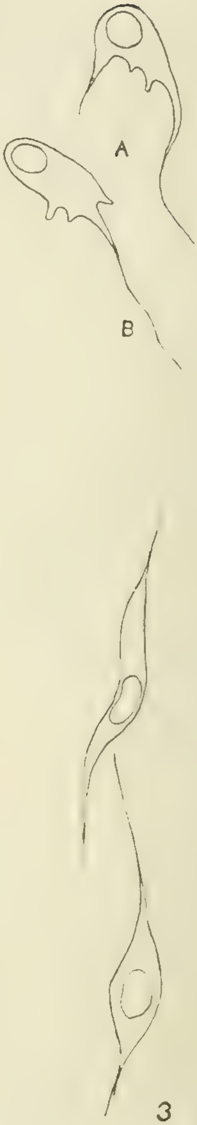


Fig. 1 A group of mesenchyme cells indicated in outline, camera lucida sketch, wandering out from the side of an early embryo, 45 hours old. Two kinds of cells are seen, one with delicate filamentous processes and another amoeba-like cell.

Fig. 2 A similar group in one microscopic field from an embryo 48 hours old, again showing the two types. The elongate spindle cells are future endothelial cells.



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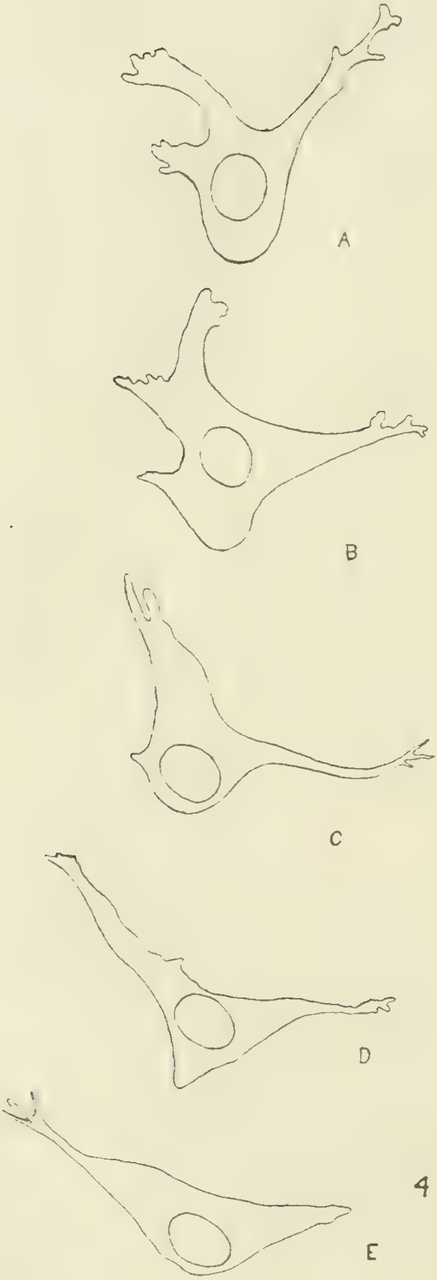

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It is, therefore, difficult to state that the spindle-shaped cells may not change to the heavier amoeboid pattern and vice versa. But we shall see that these two forms are the probable if not actual forerunners of two groups of cells later, at any rate, with permanent shapes and structures differing in much the same way as the early appearances now differ. Figure 2 again shows a sketch from life of wandering cells on the yolk-sac of a 48 hour embryo, and here as usual the spindle cells are contrasted with the amoeboid ones. The spindle cells are assumed to be future vascular endothelial cells and the amoeboid cells are probably the future chromatophores of the yolk-sac.

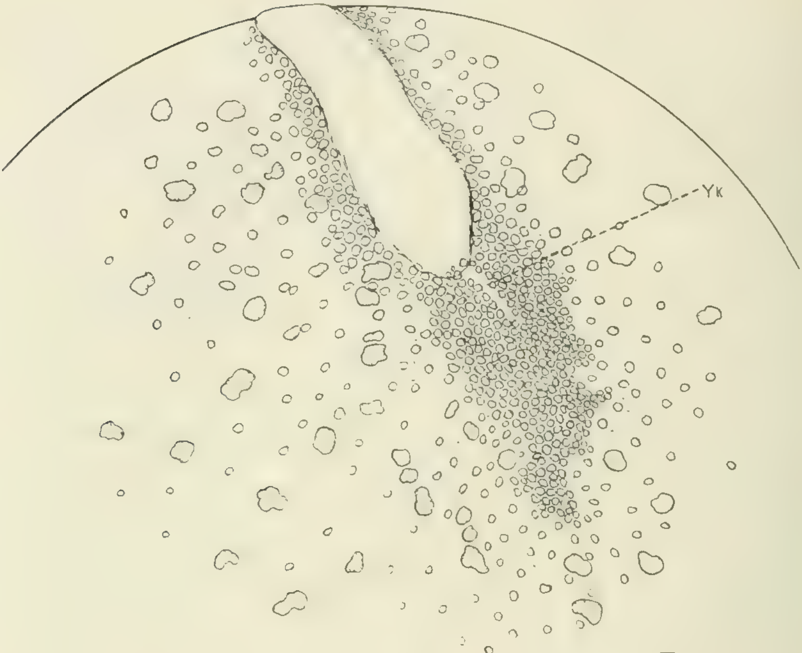
The places from which cells wander out most actively are the borders of the tail, and particularly from that mass of cells representing the obliterated germ-ring. Figure 5 shows the cellular arrangement around the tail end of a 48 hour embryo fixed and cleared. The cells have withdrawn their processes. The open space in the cell group at the tip of the tail, *yk*, is the place still remaining between the borders of the germ-ring. Later, the tail of the embryo grows over this cell group so that it is less conspicuous. Figure 8, the tail end of a 56 hour embryo, shows such a condition.

The important fact which we shall later consider is that these cells form a mass continuous with the mesenchymal cells within the tail end of the embryo. The wandering cells may be interpreted to grow out from the end-bud or blastopore lip. They are a scant rudiment of the peripheral or ventral mesenchyme usually growing away from the blastopore lip over the yolk mass in the reptile and the bird. It will be presently shown that such an interpretation is upheld by the nature of the products to which these wandering cells give rise.

Figure 6 illustrates the wandering away of cells from the lateral mesoblast of an embryo with two pairs of somites, 48 hours old. Figure 7 shows the head end of a 56 hour embryo. Scarcely any

Fig. 3 Camera outlines of wandering mesenchyme cells 48 hours old, all of the future endothelial type, highly magnified. *A* and *B* are two outlines of the same cell at a 6 minute interval.

Fig. 4 Camera outlines of one cell drawn at 5 minute intervals *A* to *E*. The cell is a migrating future chromatophore in an embryo 50 hours old (3b. DD ob.)



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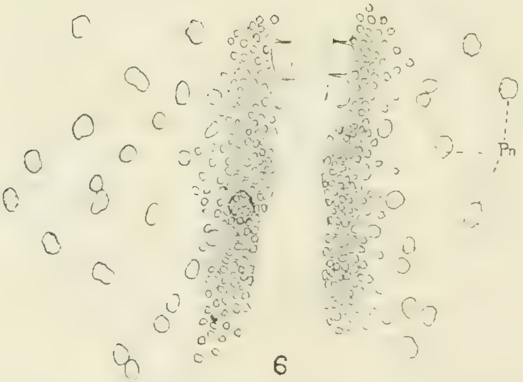

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Fig. 5 Camera lucida outline of the tail end and caudal yolk region of an embryo 18 hours old, fixed and cleared in glycerine-formalin. The germ ring just closing over the yolk pole, numerous mesenchyme cells beginning to wander away from the caudal end, huge periblast nuclei indicated in outline and stipple over yolk. *Yk*, polar bit of yolk just being covered by union of germ-ring border.

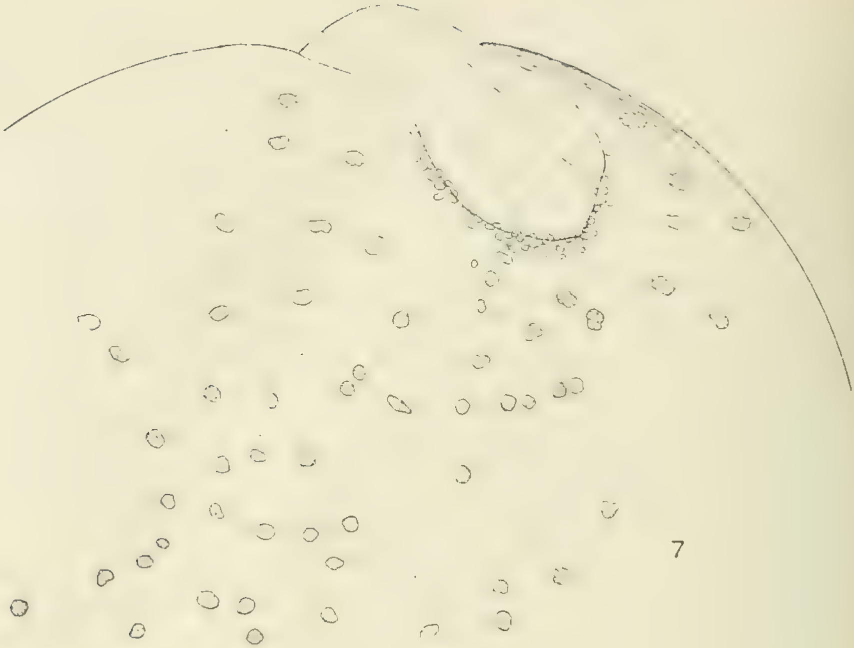
Fig. 6 Portion of the caudal half of an embryo of 48 hours showing the first two pairs of somites recently formed, cells of the lateral plate mesoderm extend out upon the yolk as shown in outline, some beginning to wander away. Periblast nuclei outlined and stippled. Glycerine-formalin specimen.

cell migration is taking place from this region. A few mesenchyme cells are found along the border of the head; these cells later take part in either the formation of the heart or pericardial wall. The tail end of the same embryo, figure 8, shows a remarkable contrast; here there is an enormous wandering out of cells from the mesoblast of the embryo. The two figures show the huge periblast nuclei to be widely distributed throughout the surface of the yolk sphere. These drawings are from cleared specimens and the cell outlines are more or less circular without the beautiful processes characteristic of the living.

The tail end of a living embryo 72 hours old, some time before the blood began to circulate, is illustrated by figure 9. The circle beneath the tail represents Kupffer's vesicle. The various shaped mesenchymal cells are represented in the act of wandering out over the nearby surface of the yolk. The embryo and yolk are beautifully transparent in life and the cells are clearly seen as they move upon the surface of the periblast.

An entire embryo, except the anterior portion of the head which extends beyond the curve of the yolk, is shown in figure 10 at a lower magnification. This specimen was 76 hours old when drawn. The heart had begun to contract slowly and feebly but no circulation of fluid had begun. Groups of mesenchymal cells are seen wandering away from the lateral and particularly the caudal regions of the embryo and are now scattered broadly over the yolk surface; there being very few, however, in the anterior region. The lateral plates of the mesoderm are seen at the sides of the head, and a circle at the caudal end indicates the Kupffer's vesicle which is always clearly shown at this stage.

In embryos of 72 hours, and somewhat earlier, there are wandering out from the tail region a number of cells slightly smaller than the two types mentioned above. These small cells tend to be more or less circular in outline but show slow amoeboid movement as they send out short blunt processes. They group themselves into small clumps and are to give rise to erythroblasts or future red blood corpuscles in the yolk-sac as shall be discussed beyond. Figure 31, page 569, shows six such cells from the living yolk-sac of an embryo 90 hours old;



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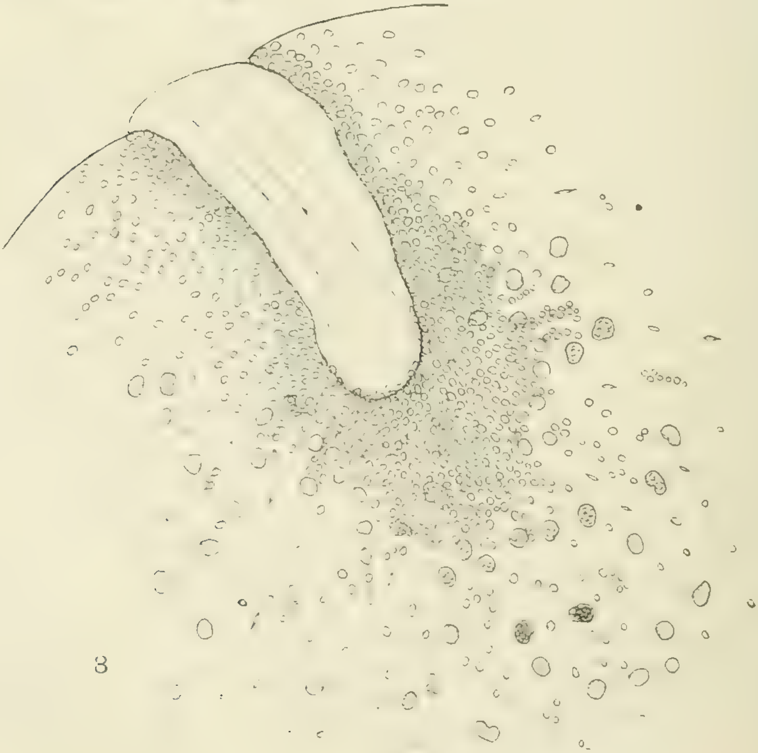

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a circulation was partially established in this specimen but these cells had not yet been taken into the vessels.

The various wandering cells then represent the mesodermal layer of the yolk-sac in the teleost. They never assume a membranous layer-like arrangement, but finally differentiate into the characteristic structures of the yolk-sac. As is shown by the illustrations, these cells are very numerous and during their earlier stages are actively changing their shape and moving over the yolk surface.

We may now consider the further development of such cells the living embryos.

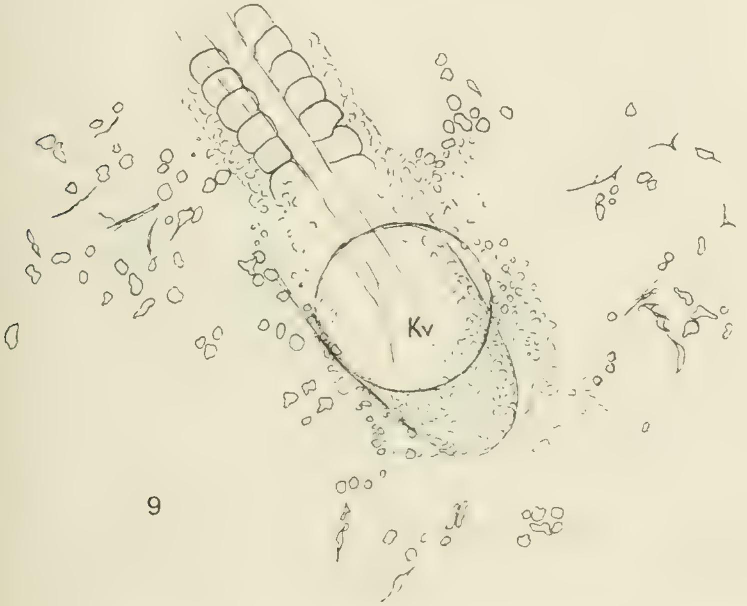

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Fig. 7 Outline of the head end of a 56 hour embryo, scarcely any wandering mesenchymal cells in this region. Large periblast nuclei scattered over yolk surface.

Fig. 8 The caudal end of the same embryo; note the great contrast in the abundance of out-wandering mesenchymal cells. Glycerine-formalin specimen.

Fig. 9 The caudal end of a living normal embryo of 72 hours, with beautifully delicate mesenchymal cells wandering away from the body; *Kv.*, Kupffer's vesicle (3b. 2/3 ob.).

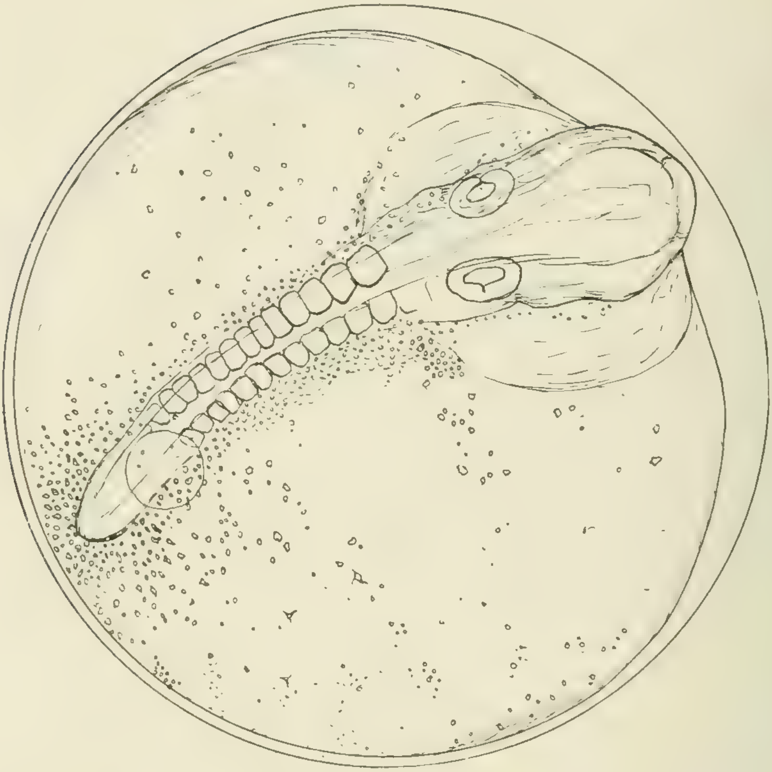


Fig. 10 A camera sketch of an entire embryo of 76 hours except the anterior end of the head. The mesenchymal cells wandering away from the tail region and to a less degree from the sides of the body.

DEVELOPMENT AND DIFFERENTIATION OF THE WANDERING CELLS

1. *Chromatophores*

a. The black type of chromatophore. The first to be considered of the four types of cells which develop on the yolk-sac are the black chromatophores. These are the largest and most conspicuous cells of the yolk-sac. In the early stages discussed above, one notes even in embryos of 2 days that certain cells of the yolk mesenchyme are considerably larger than others.

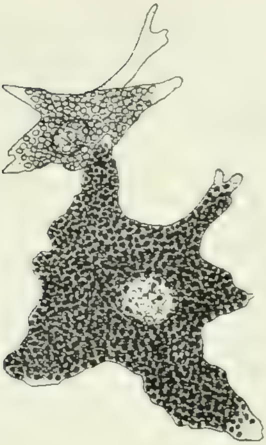
These large cells may be followed through their development and they will be found to differentiate into one or the other of the two types of chromatophores. The amoeboid cell shown in different stages of movement already referred to as figure 4, from a 52 hour embryo, is of this large type, and concluding from my observations on great numbers of embryos, this is an early condition of the future black chromatophore before any pigment granules are deposited.

Slightly older stages, figures 22 and 26, show the same cells containing a light amount of pigment granules. Between the second and third days the pigment granules appear and in an embryo 72 hours old, end of the third day, the chromatophores are already well differentiated freely moving huge cells.

A black chromatophore from an embryo 72 hours old is shown in figure 11 with one of its processes overlying the body of a brown chromatophore, the type to be considered in the following section. The black cell is loaded with coarse granules. The nucleus occupies a central position and is clearly shown on account of the displacement of the pigment granules by its transparent body. Several pseudopod-like processes project from the chromatophore which is actively moving. The clear cytoplasmic tip of the pseudopod extends beyond the granular mass.

Figures 12 and 13 are two other illustrations of the same cell after 15 minute intervals. Its shape is constantly changing and it is slowly moving in a direction towards the right side of the page. The brown pigment cell is also moving and their rates of progress are indicated by the increasing distance between them.

This movement of the chromatophores continues until about the end of the fourth or middle of the fifth day in the normal embryos. By this time all of the black pigment cells of the yolk-sac with few exceptions have taken up more or less permanent positions along the walls of the blood vessels or around the surface of the pericardial space. The individual chromatophores have increased enormously in size as is seen by comparing figures 11, 12 and 13 with figure 14, all drawn at the same magnification, though figure 14 is one-third more reduced in reproduction.



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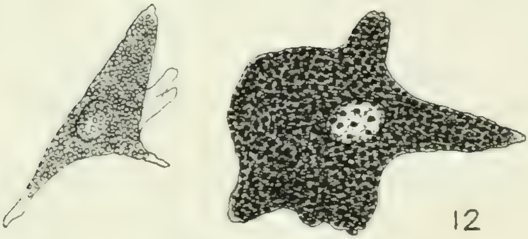

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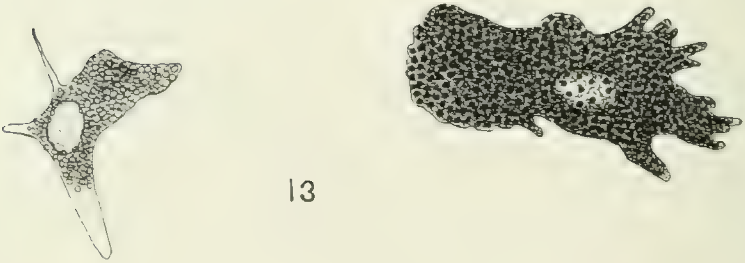

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It must be appreciated, however, that some of the difference in extent is due to the flattening of the cells in figure 14.

Figure 14 shows two huge pigment cells on the yolk-sac of a 5 day embryo in the act of arranging themselves along a vessel wall. The granules are not so densely arranged as in the younger stages, since the cell body is greatly thinned out in pressing around the vessel. A number of granules are often arranged in solid black lines and masses as indicated in the figure.

The two cells are close together and a very peculiar phenomenon is taking place. Each cell sends out short processes to meet similar processes from its neighbor. The processes fuse, and finally the two cell bodies melt into one thus forming a pigmented syncytium about the vessels of the yolk-sac. The syncytia continue to expand along the vessels as enclosing sheaths (fig. 15). The dense black of the young chromatophores becomes a steel grey as the granules are more thinly spread along the vessels.

In order to test whether the cells had actually joined or fused to form a true syncytium, I attempted to contract them, thinking that this should pull them apart unless they were actually united. The various solutions of KCl which Dr. Spaeth has found to contract the chromatophores within the embryo's body failed entirely to produce any change in the chromatophores of the yolk-sac. Solutions of adrenalin of one to 1000, one to 10,000 and one to 100,000, which Dr. Spaeth so kindly supplied me, were then tried. These solutions contract the pigment cells on the brain of the embryo until they appear as small black dots, but neither the black nor brown chromatophores on the yolk-sac respond in the slightest degree. Such specimens were preserved to show the extreme contraction of the chromatophores over the brain of the embryo in contrast to the unchanged pigment cells of the yolk-sac.

Fig. 11 A black and brown chromatophore lying in contact on a yolk-sac of 72 hours. The black cell is much the larger with broader pseudopod-like processes; both are in active movement as shown by comparing figure 12, of the same two cells 15 minutes later and figure 13, the same cells 20 minutes after figure 12. (3b. DD. ob.).

From this it would seem as though the material of the chromatophore had lost its contractile or wandering power after once becoming arranged around the yolk vessels. Those black chromatophores which retain their cellular individuality along the borders of the pericardial space also fail to contract when treated with KCl or adrenalin.

Although this physiological test failed to serve the purpose for which it was used I feel certain, after many observations, that the black chromatophores actually do form true syncytial masses as they surround the vessels.

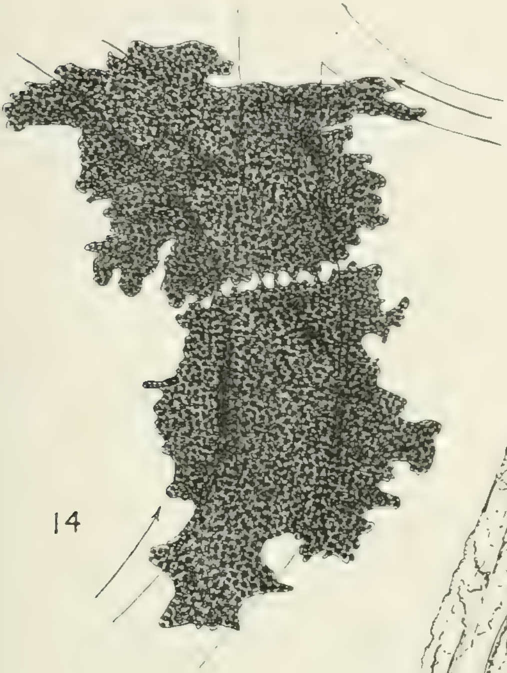
b. The brown type chromatophore. The brown chromatophores differentiate on the yolk-sac at about the same time as the black. They are always somewhat smaller and more delicately formed cells than the black, and react in a slightly different manner. Figures 22 and 26 show several brown chromatophores before the end of the third day. They are paler in appearance and more elongate in shape than the black cells.

The two types of cells are well contrasted in figures 11, 12 and 13 referred to above. The brown cell is smaller, with more delicate processes and is the more rapidly moving of the two. The three figures indicate its condition in embryos of 72 hours.

These pigment cells also wander to the vessel walls and yolk spaces and take on their permanent condition about the fifth day. Figure 16 illustrates one of the exquisite brown pigment cells in a yolk-sac of 5 days. The nucleus is still distinguishable in life while it is not in the black cells of this age. The mossy branched processes projecting from all sides give to this cell a most fascinating form.

Fig. 14 A camera lucida drawing of two huge black chromatophores lying upon a yolk vessel of a 5 day embryo. The adjacent sides of the chromatophores are beginning to fuse to form a syncytium. The direction of blood flow is indicated by the arrows.

Fig. 15 A syncytial mass of black chromatophores forming a sheath about the vitelline vessels. The chromatophores become so thin that the pigment granules are spread apart giving a less intense color. The individual cells are completely lost in the syncytium (3b. DD. ob.).



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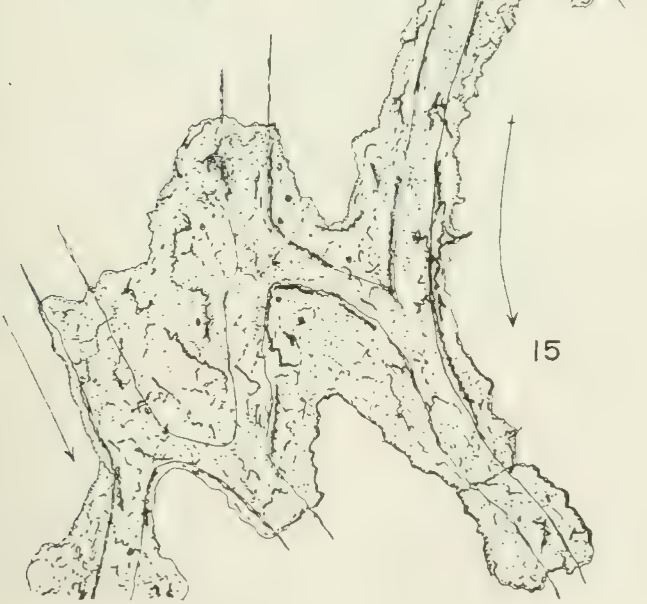

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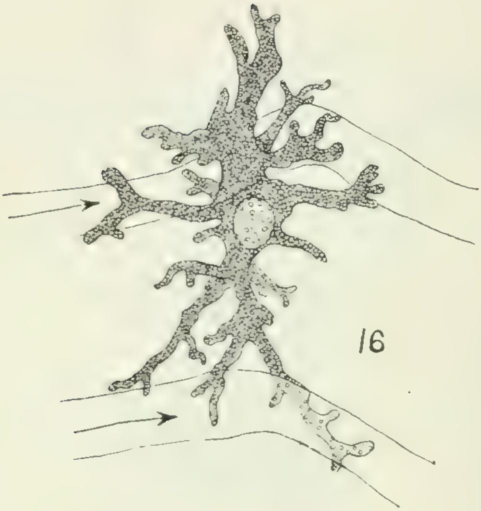


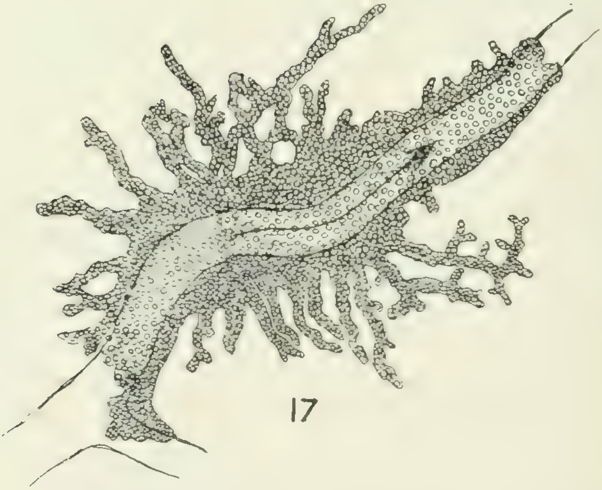


Fig. 16 A brown chromatophore on the yolk of a 5 day embryo. The cell is coming in contact with two vessels shown in outline. The moss-like processes extend from all sides of the cell.

Fig. 17 A similar cell 12 days old surrounding a yolk vessel. The complex processes from this cell are quite in contrast to the almost smooth border of the black chromatophores of figures 14 and 15. The brown cells never fuse to form syncytia.

Finally, in older embryos the cell body often surrounds a vessel, as shown in figure 17, but the processes persist and project from it in all directions, forming a striking contrast to the more or less smooth outlines finally assumed by the black cells, figures 14 and 15, as they surround the vessels.

The brown chromatophores do not group themselves together or form a syncytial mass as the black pigment cells are prone to do. They remain individually separated and many really never become associated with vessel walls, but lie scattered on the yolk surface.

In early embryos, from 72 to 90 hours, the brown pigment cells may sometimes, though rarely, get into the blood stream. I have never been able to observe one in the act of entering the current. Yet in a quiescent state they might become surrounded by endothelial cells along with the erythroblasts, and finally be swept away. They might, on the other hand, actually migrate through the porous wall of an early vessel.

The enormous brown pigment cell presents a smooth circular outline as it is carried along in the blood current. On account of its size the chromatophore often meets with difficulties in passing narrow portions of the vascular system. Several such cells were seen in the blood circulation of different embryos during the course of the observations, and when once located in the current the same cell could be seen periodically for a long time as it came around again and again through the vesssel within the field of study. There is no question of the identity of these cells, as their characteristic reddish brown color and coarse granular structure is readily recognized. It is most improbable to think of them as becoming changed into any type of blood corpuscle, and it is doubtless entirely by accident that they occasionally become entrapped within the vessel wall and washed away by the current.

c. Behavior of the chromatophores in specimens with no circulation. The behavior of both the black and brown types of pigmented cell is distinctly different in embryos without a circulation of the blood from that described in the two previous sections for normal embryos.

During the early stages, up to the beginning of the fourth day, the cells wander in amoeboid fashion much the same as in ordinary specimens. In other words, at this time the condition is the same in all embryos since the blood has not begun to circulate in any. At about 72 hours the blood circulation begins in the normal embryos and the pigment cells seem to be attracted to the vessel walls, as already pointed out. If the circulation does not begin at this age, the plasma accumulates in various spaces, chiefly the pericardial sac and Kupffer's vesicle at the caudal end of the embryo. The excessive accumulation of plasma in these spaces causes them to be in many cases hugely distended. The heart in such specimens also becomes a saccular structure filled with plasma which it is unable to pump on account of one or another deficiency in the vascular system.

Large numbers of chromatophores of both types tend to aggregate about these plasma filled spaces and partially cover their walls. The spaces are thus rendered more conspicuous. In some specimens this coating of the distended plasma sacs by pigment cells is most remarkable, but such an arrangement is not invariable and in a number of individuals the pigment cells are irregularly scattered over the yolk-sac with no recognizable pattern or system.

The heart of embryos in which there is no blood circulation is almost without exception covered with chromatophores. These cells often form a perfect sheath about such hearts whether the heart is a plasma filled sac or a mere string. The patterns of these arrangements are illustrated by numerous figures, particularly figures 15 to 20 in the previous paper.

A point of much interest in this connection is the fact that the heart of the normal embryo is entirely free of pigment cells.

The behavior of the chromatophores of the yolk-sac in normal individuals where they tend so decidedly to arrange themselves along the blood vessel walls along with their affinity for the plasma filled spaces in the non-circulating condition would seem to indicate that the chromatophore was attracted by the plasma itself, or some element which it contains. The distended plasma filled heart in the non-circulating cases is covered with pigment as would be expected, yet the solid string-like heart present in many such specimens is also covered with pigment though it of course is entirely empty of plasma. In this last case, however, the string-like heart actually stretches as an axis through the pericardial space which is distended with fluid. The cells arrange themselves around the wall of the pericardium and on reaching the venous end of the heart migrate along it and so cover the heart string or tube in their effort to come in close proximity to the plasma. The distended condition of the pericardium may in this way account for the pigment arrangement along the heart in the cases with experimentally arrested circulation.

The normal heart is constantly pumping the plasma through itself, yet pigment cells are never present in its wall since they are all arranged along the vessels of the yolk-sac. In non-circulating cases many vessels form on the yolk-sac and some become quite well developed, while others actually surround the blood corpuscles of the yolk islands. Such vessels are at times covered with pigment but probably through accident as the pigment is irregularly scattered over the entire yolk-sac. Yet the pigment cells on such vessels never arrange themselves in the definite sheath-like fashion characteristic of the vessel pigment in the normal embryo.

Figure 18 illustrates the lack of arrangement of the chromatophores on the yolk-sac of a 16 day embryo without a circulation; compare figures 15 and 17 of pigment in the normal embryo. Figure 35, see beyond, also illustrates in a striking way the irregular grouping of black chromatophores in the neighborhood of a collection of stagnant blood islands in an embryo of 14 days that never had a circulation of its blood.

All of these reactions cause one to wonder what is the actual function of the pigment cells upon the yolk-sac. The entire egg is rather transparent and their function might be to protect the vessels from the light, yet the vessels are never completely covered and the development of the eggs in the dark is normal but not in any way supernormal.

The pigment cells form such a complete sheath about the vessels in some cases that one might be led to imagine that their expansion and contraction would serve as vaso dilator and contractor. Yet when they are along the vessel wall I have failed to see them contract or expand even when treated with substances such as KCl and adrenalin that violently contract the chromatophores within the embryonic body. These experiments have not been carried sufficiently far since they were directed towards another point, still they indicate at least that the pigment sheath of the vessel wall does not respond as a delicate vaso-motor apparatus.

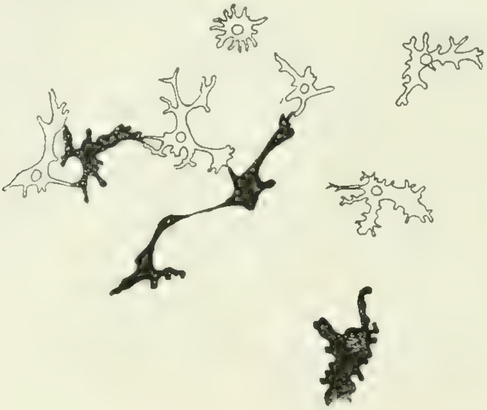


Fig. 18 A group of brown, indicated in outline only, and black chromatophores on the yolk-sac of a 16 day embryo in which the blood has never circulated. There is no arrangement of the pigment cells on vessels and no real syncytium of black chromatophores as compared with the conditions in the normal embryo

Wenckebach ('86) found in certain pelagic eggs containing a number of oil drops which invariably floated up that pigment cells often completely surrounded the oil globules, and as he thought prevented these globules from focussing heat or light on parts of the embryonic body. The oil drops in the demersal Fundulus yolk do not particularly attract the pigment cells and they are rarely found to lie against the oil globule.

The function of the pigment in the yolk-sac of Fundulus, if it has any function other than its own existence, is most difficult to determine. The same is true of the abundant pigment in the coelomic wall and other internal structures of many animals.

d. Relationship of chromatophores to blood and endothelial cells. There has been much discussion in the literature regarding the relationship of the chromatophores to blood corpuscles and to endothelial cells. The actual relationship of these cells is clearly brought out by a careful study of the living yolk-sac in Fundulus. The cells are completely different and their structures when once established are consistent in their particular type.

The black chromatophores, the brown chromatophores, the endothelial cells and blood corpuslces are all derived from mesenchymal cells which wander away mainly from the caudal region of the embryo during early stages of development. These cells come to lie in the primary segmentation cavity of the yolk-sac beneath the ectoderm and over the periblast syncytium. The mesenchymal cells very soon begin to show certain differential characters in structure and behavior. When certain ones of the cells incline in a definite direction their development progresses continuously along this line.

Observations on the normal living embryos and comparison with those individuals without a yolk-sac circulation lead one to conclude as follows regarding the wandering mesenchymal cells. At the time these cells leave the embryo proper to wander over the yolk, differentiation has proceeded to some extent in the embryo and the mesenchyme cells are probably somewhat limited in their potentialities. Certain of them are derived from the same portion of mesenchyme that gives rise to the intermediate cell mass or future red blood cell forming mass within the embryo. This mass is located towards the caudal end of the embryo and the wandering cells derived from it finally come to lie on the posterior and ventral surfaces of the yolk-sac and form islands of red blood corpuscles. Few if any of these cells reach the anterior regions of the yolk-sac before the circulation of the blood begins. In embryos that *never* have a circulation the blood islands lie beneath the tail of the embryo and on the ventral yolk surface. The future endothelial cells wander out from the caudal end and side of the embryonic body and finally line up to form vessels in a manner to be described beyond. The pigment cells also wander out from the lateral regions and differentiate into chromatophores of either the black or brown variety.

It would seem that these cells must have some potential differences at the time they come to lie in the yolk-sac, since from that time on they all appear to be in an identical environment. Two cells lying side by side in the yolk-sac above the periblast and beneath the ectoderm would be expected to develop and grow in similar fashion unless there were some internal difference between them. I have thus concluded that the mesenchymal cells which wander in the yolk-sac of the Fundulus embryo must be potentially of four different classes when they first wander out, although all have the ordinary appearance of embryonic mesenchymal cells. Otherwise, it is difficult to conceive why they should develop into four distinct types of cells while all are surrounded by an identical environment so far as is possible to discover. Differentiation in various directions must be due either in the first place to similar cells developing in different chemical or physical surroundings, or in the second place it may result from potentially different cells developing under identical conditions.

The four types of cells are all derived from mesenchyme, just as the thyroid follicles and pulmonary epithelium are derived from endoderm but from different endodermal anlagen, and further than this there is no relationship. Pigment cells and blood corpuscles are perfectly separate and distinct types derived from different mesenchymal analgen and are not in any way transmutable.

2. *History of the endothelial cells*

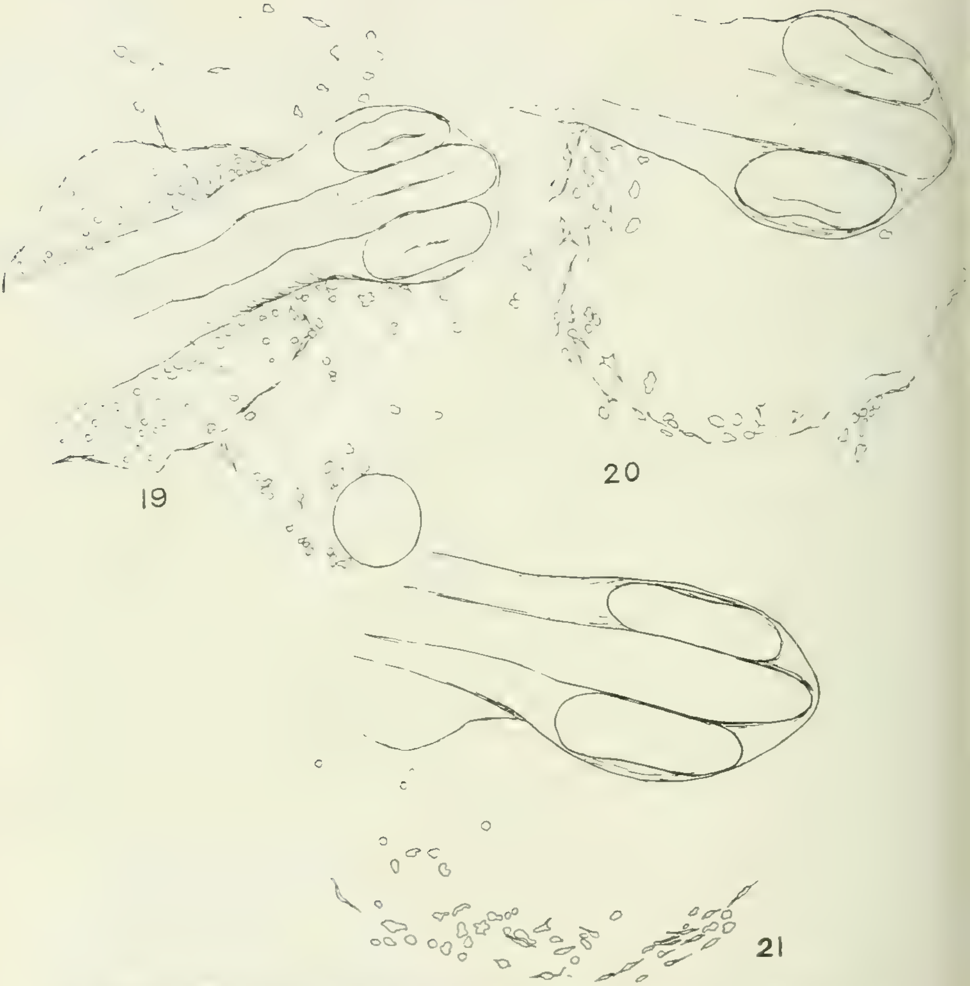
The endothelial cells on the living yolk-sac of Fundulus embryos are readily recognized. Their entire behavior in the formation of the earliest yolk vessels may be traced in a manner to fully repay the patient observations necessary in order to follow through the processes.

Among the early wandering cells migrating away from the lateral borders and caudal end of the embryo it is noted that certain ones assume a delicate spindle shape with filamentous processes extending from their ends and occasionally projecting from their long sides. In a 48 hour embryo these cells already present the appearance of individual endothelial cells. They migrate indefinitely for a few hours and then tend to group themselves in more or less irregular collections.

Up to this time no one from mere observation could be absolutely certain that the cells of this rather characteristic appearance are actually to become vascular endothelial cells in all cases. The possibility, of course, exists that the elongate spindle cells may at times round up and then assume the more amoeboid shape of the probable future chromatophore. Yet since the shape of these cells is so characteristic and such shapes are so constantly present, one is inclined to believe that the same cell may actually retain this character until it really becomes a component part of a vascular endothelial arrangement.

Figures 19, 20 and 21 illustrate the region along the side of the embryo's head at 48 hours old. It is in this region that the first large yolk vessel develops. This vessel carries blood from the body of the embryo around a short circuit to reach the venous end of the heart and thus in a way relieves the flow that otherwise would force itself through the small poorly formed vessels in the embryonic body. This vessel is, therefore, of necessity one of the earliest to develop. The three figures, 19, 20 and 21, show variations in the arrangement of the wandering mesenchymal cells in the region of the future vessel.

In figure 19 there is really no definite cell aggregation except along the edges of the head mesoblast as it spreads somewhat over the yolk, yet a few of the cells show the typical spindle shape. Figure 20 indicates a tendency of the mesenchymal cells to line themselves in a group exactly along the course of the coming vessel. Many of the cells in this group give the actual appearance of an endothelial cell after it is fully developed and forming one of the units in a vessel wall. The embryo illustrated by figure 21 shows much the same condition. Very few mesenchymal cells occur between this cell aggregation and the side wall of the head. Lateral of the vessel group the cells are also not numerous and have no system of arrangement.



Figs. 19, 20 and 21 Outlines of the head regions of three living embryos from 48 to 50 hours old, showing different conditions in the grouping of mesenchymal cells on the yolk which later give rise to the large vessel that short circuits blood from the side of the embryo around over the yolk to the venous end of the heart. The future vessel wall is now separate spindle shaped mesenchyme cells.

This cellular aggregation may then be regarded as the actual anlage of the vascular endothelium of the future vessel. The anlage consists merely of a group of separate wandering mesenchyme cells, and not of a capillary net in any sense.

A slightly older embryo shows a still more definite alignment of the mesenchymal cells and still later presents the appearance of cellular strings or cords as illustrated in an embryo of 67 hours by figure 22. Here the wandering mesenchyme cells have differentiated to such an extent that they are readily distinguishable as black and red chromatophores and elongate endothelial cells.

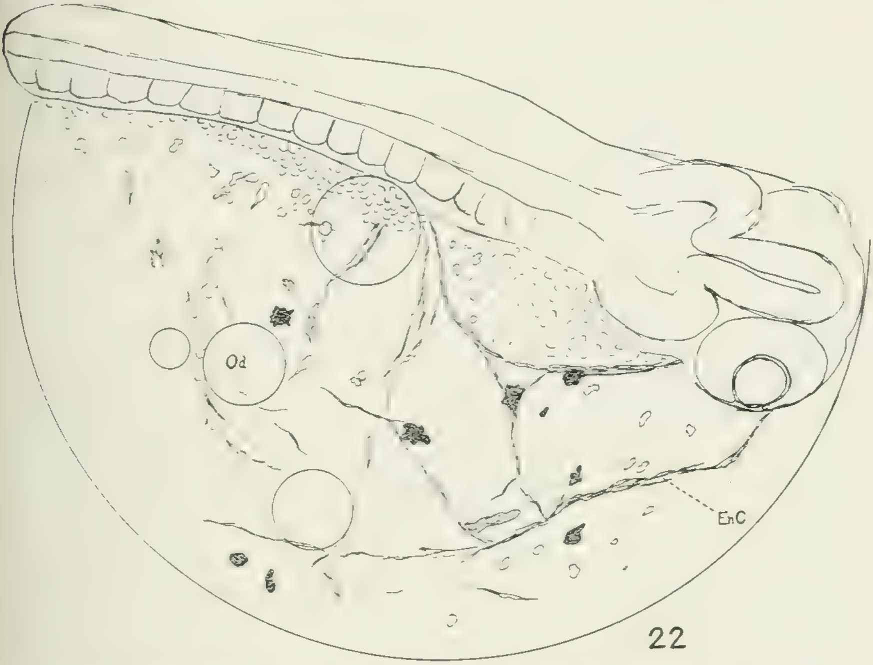


Fig. 22 A sketch of a 67 hour embryo showing the stage in the origin of yolk vessels in which the mesenchymal cells have a linear arrangement. Early black and brown chromatophores are also shown in the yolk-sac. *Od*, oil drops.

Early erythroblasts are also seen on the caudal region of the yolk-sac in such an embryo, but are not shown in the aspect here illustrated.

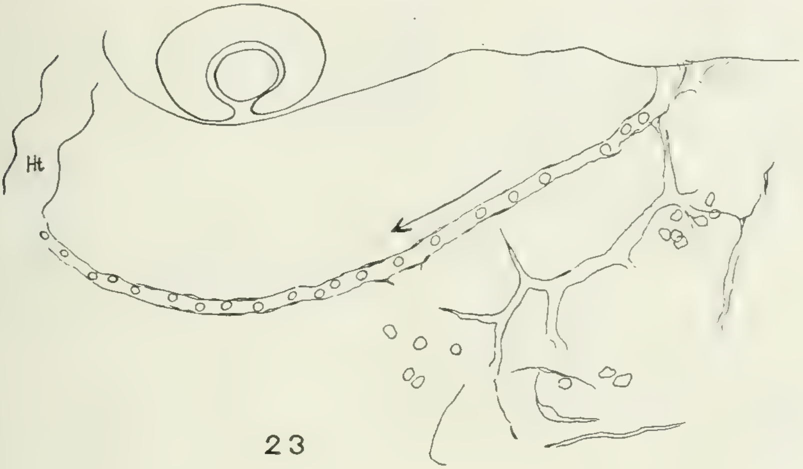
The endothelial cells are strung out in various directions and several linear groups are more or less isolated from the rest. The string to be the future large vitelline vessel is not clearly continuous posteriorly, but anteriorly it is well outlined extending towards the venous end of the now forming heart which has not yet begun to pulsate. Here there is no further doubt that these elongate spindle cells are the elements which will make up the endothelial lining of the vessel wall.

There is considerable variation in the rate of development of the yolk vessels in embryos of the same number of hours. Some individuals may be in the condition just described, while others of this age may have already begun to establish a circulation of the blood. Figure 23 shows the yolk region lateral of the head in another embryo at 67 hours. In this specimen an incipient circulation has begun and the cord of cells illustrated in figure 22 has now become a small hollow tube sufficiently open to allow the passage of a single file of corpuscles from the side of the embryo around to the venous end of the heart. The individual cells composing the vessel are distinctly seen and their nature is clearly made out with a higher magnification. They retain the same general appearance presented before entering into the vascular arrangement.

Near this vessel is shown in figure 23 a partially formed vascular plexus which is broken in several places and entirely disconnected from the large vein through which the blood is flowing. There is of course no circulation of fluid in this partially formed plexus.

Figure 23 was sketched with a camera lucida at 12 M. and about three hours later at 2.45 P.M., figure 24 was sketched from the same field. The main vessel in accord with Thoma's ('93 and '96) first law of vessel growth has increased in calibre on account of the increased flow and pressure of the circulation. It now permits the passage of three or more corpuscles abreast and is a strongly developed vessel. The former disconnected vascular plexus has grown towards the large vessel and two of the projections shown in figure 23 have now met the wall of the vein and joined with it. One of the first corpuscles from the circulation to enter the plexus is shown in the figure tightly held in the small vessel. Immediately opposite this vessel a sprout is seen growing away from the wall of the large vein.

Figure 25 illustrates the state of arrangement at 6.00 P.M., three hours older than figure 24. The third process from the



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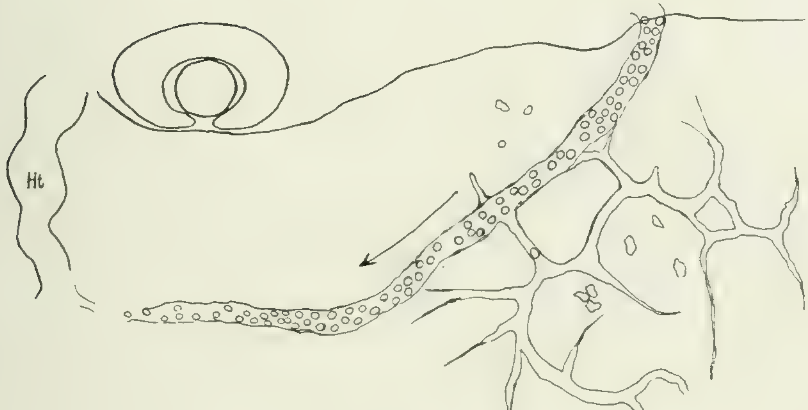

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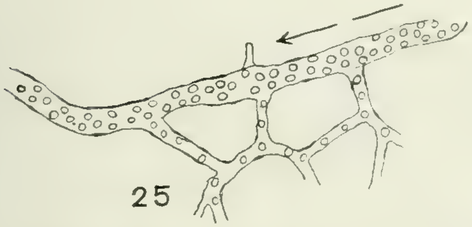

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Fig. 23 The large vitelline vein in an embryo of 67 hours just beginning to permit the passage of blood through its lumen. Corpuscles moving in single file. This becomes the largest vessel of the embryo and arose from the arrangement of the freely wandering mesenchymal cells of figures 19, 20 and 21. In the

plexus has here joined the vein and corpuscles are freely passing into the vessels. The sprout from the vein wall is still seen opposite the entrance of the middle vessel. The small plexus arose in loco entirely independently and subsequently became connected with the larger vessel which also arose as we have seen from a group of mesenchymal cells.

Figure 26 shows another lateral view of the head region of an embryo of 67 hours in which the large vein is filled with circulating corpuscles and the beginning of the same plexus followed in figures 23, 24 and 25 is seen lateral of the vein. At this stage the plexus is entirely disconnected and separated from the vein.

In the formation of the large yolk-sac vein, as well as all other vessels arising upon the yolk, there is nothing to be seen of the forerunning capillary plexus so strongly emphasized by Thoma in the yolk-sac of the chick. There is no selection and dilation of certain channels in the capillary plexus of the teleost's yolk-sac to form the veins. Here the veins seem to arise in rather definite localities and soon expand into their full form after the circulation has become established.

This method of the formation of vessels was beautifully brought out by Wenckebach ('86) in the early study already referred to so often. He concludes: "Aus diesen Beobachtungen geht hervor, dass Mesoblastzellen durch selbständige amoeboïde Bewegungen die Wände der Blutgefässe des Dotters bilden." Raffaele ('92) later confirmed this observation and further was strongly of the opinion that in selachians and other vertebrates a similar process of vessel building from wandering cells also takes place.

From my present studies on the normal and abnormal Fundulus embryos, I can see no way to doubt that the endothelial wall of the primary yolk vessels in the bony-fish is formed by arrangements of wandering mesenchymal cells.

lower part of the camera sketch is shown an independent capillary plexus not yet connected with the vein. *Ht*, heart.

Fig. 24 The same vessels 2 hours and 40 minutes later. The main vessel has increased in caliber and two branches of the capillary net have joined the vessel.

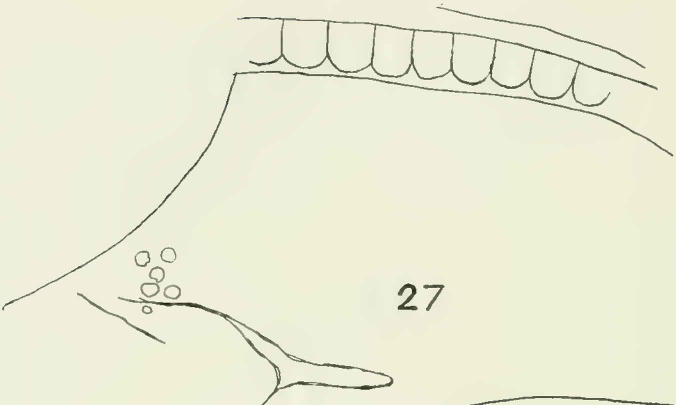
Fig. 25 The same vessel three hours later, a sprout is given off opposite the union with the middle capillary and corpuscles now enter all three capillaries. The arrows indicate the direction of blood flow.

Wenckebach's description cannot be fully agreed with in all detail. He thinks, for instance, that cells forming part of the vessel wall are brought by the blood stream. These cells have small protoplasmic processes but they are not in any way to be confounded with the "definitiven Blutkörperchen." Such cells have never been observed in the Fundulus embryos and if they exist, which is very improbable, their part in vascular formation is extremely insignificant.

Wenckebach observed the three primary vessels on the yolk to bud and give off sprouts forming other vessels. The wandering cells also formed small separate tubes which later became connected to form a portion of the vascular net. By these methods the complex vascular net of the yolk-sac was finally formed. This agrees closely with what may actually be seen to occur in the embryos of Fundulus.

Considerable variation occurs in the position of vessels and a number of actual abnormalities are found in which the bilateral arrangement is completely disturbed. These abnormalities are frequently very instructive for a thorough understanding of the origin and development of the yolk-vessels. Occasionally, a group of cells will form a completely isolated endothelial space which may fail to associate itself with a vessel. Figure 27 shows such an isolated space in a yolk-sac of 90 hours old; solid endothelial tips project from the space, yet it is completely isolated so far as can be determined with the highest power, and at the same time every part of it is clearly and distinctly seen.

When the early yolk vessels are studied under high magnification, the individual cells may be clearly observed and they are strikingly the same as before they became associated to form the vessel. The cells are not closely arranged but distinct intervals and spaces exist between them and filamentous processes often project far into the lumen and may actually at times fuse with a similar process from a cell on the opposite side of the wall. These filaments thus stretch across the vessel and may even persist after the blood has begun to flow. They are well seen by focussing so as to get an optical section through the cavity. Cor-



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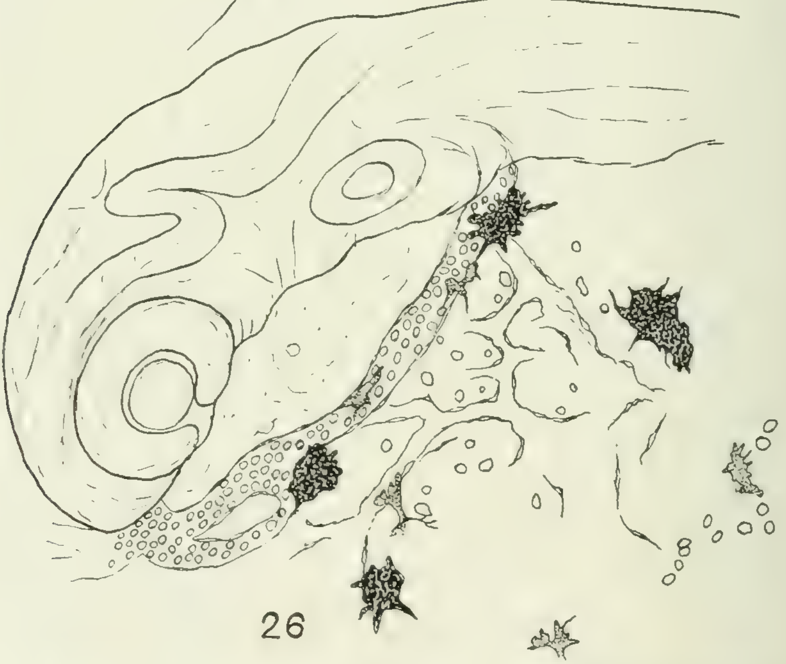

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Fig. 26 Outline of the yolk vascular condition in the head region of a 67 hour embryo. Blood is circulating freely in the large vessel but the yolk net of vessels is not yet connected with the current. Early black and brown chromatophores are also shown.

Fig. 27 An isolated endothelial cavity with solid projecting tips, no connection can be seen with any other vascular spaces. From an embryo of 90 hours.

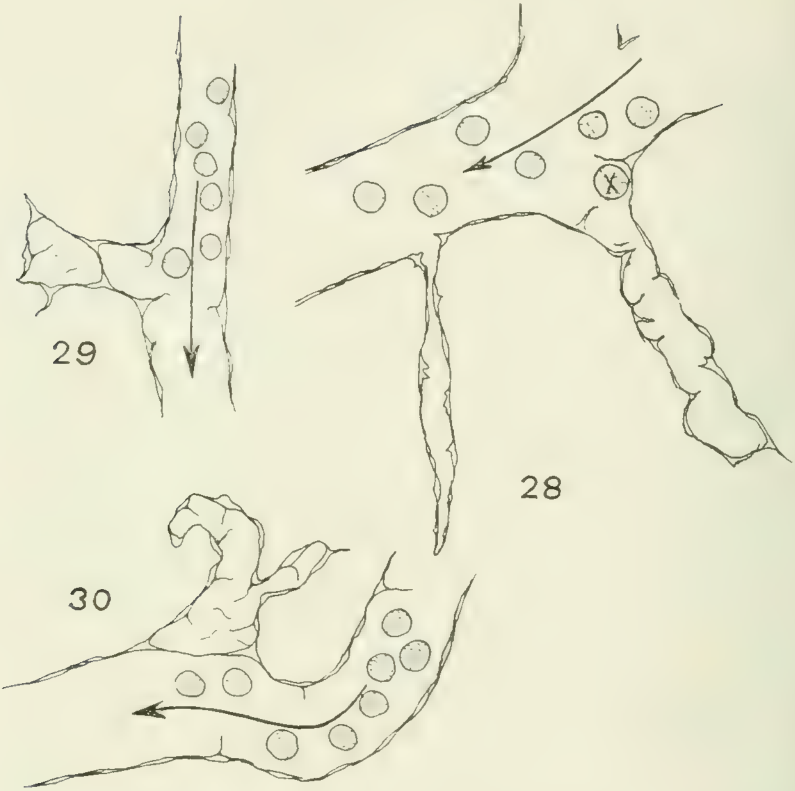
puscles often strike against the cell processes and cause them to wave back and forth as the current flows past.

The cells of the vessel wall thus maintain much of their individuality and may actually separate themselves or loosen away from the small growing tip of a vessel. The tips of the vascular sprouts probably break up or disassociate in this manner to include small groups of corpuscles which may be seen to enter the vessels from the yolk surface.

A most instructive specimen for a study of the cellular elements of the vascular endothelium is one in which the circulation has just begun. The vessels in such an embryo are still growing in length and sprouting off branches rather actively. Figure 28 illustrates such a vessel with its incipient branches. Corpuscles are passing through the vessel in the plasma current; one of these, X, is seen harbored behind an endothelial cell at the base of the outgrowth to the right. This corpuscle remained in position for more than one hour, being protected from the current by the projecting endothelial process. Such a condition is frequently seen and conveys some idea of the actual irregularity of the vascular wall.

The cells constituting the walls of the outgrowths from the vessel are changeable in shape and doubtless move their positions to some extent. The cells at the tip of the growing sprout may be seen to send out processes as if they were actually creeping along. The behavior of these vessel walls is strikingly similar to that which Clark ('09 and '12) has so clearly described for the growing lymphatics in the tail of the tadpole.

Figure 29 shows a similar vessel with a projecting bud. The cells of this bud are seen to exhibit a most indefinite arrangement; their processes project across the space and join the cells of the opposite side and none are completely elongated or flattened as are the cells of the main vessel wall. The tip cells might still be described as stellate mesenchymal cells. The appearance shown in figure 30 is much the same. The walls of these early vascular buds are extremely loose membranous arrangements with irregularities in their surfaces and openings and spaces between the cellular components.



Figs. 28, 29 and 30. Portions of vessels from three yolk-sacs of 6 day embryos. The vessels show blind endothelial sacs projecting from their walls. The constituent cells of these sacs are distinctly seen, and still retain their wandering mesenchymal characters. Filamentous processes from these cells may extend entirely across the lumen and fuse with processes from the cells on the opposite side of the wall. Corpuscles are often entangled in the filaments as well as the spaces between the endothelial cells. *X*, a resting corpuscle harbored behind an endothelial projection.

These porous or incomplete endothelial walls permit the blood cells to occasionally escape from the vessel cavity and become free within the space of the yolk-sac; or, on the other hand, a growing vascular tip may be observed at certain stages to come in contact with a group of erythroblasts, or actually a blood island unsurrounded by vascular endothelium. The tip of the vessel seems to disorganize to some extent and its cellular elements slowly surround the group of corpuscles which are later taken into the circulation as the current becomes established in the including vessel.

Unfortunately, I have never been able to observe the consecutive steps in any one case of this kind, so that an absolutely positive statement cannot be made at present. Yet numerous observations of the contact of vascular sprouts with groups of uncovered corpuscles and the ends of such sprouts containing corpuscles, as well as other arrangements, would indicate that the behavior of the endothelium in surrounding the naked groups of erythroblasts on the yolk-sac probably takes place about in the manner just outlined.

Figure 32, page 568, illustrates a highly magnified field on the yolk-sac of a 90 hour embryo. This field shows a very interesting composite of the vascular condition at such an age. The rapidly flowing blood current is freely passing through the vessel on the right. A short circuit is forming across below the curve of an arch in the vessel. This small vessel permits only a single line of corpuscles to pass. At this time only one corpuscle has entered and it is caught in the narrow tube. This corpuscle remained fixed for a long time and so enabled a comparison of its structure and appearance with the erythroblasts forming the group just below the huge black chromatophore. The cells of this group are uncovered by endothelium. On the left of the figure a portion of a vascular net not yet connected with the circulating current presents the typical appearance of an early blood vessel formation. The individual cells are loosely associated and the tip projecting towards the right slowly changes position. This tip later approached the group of erythroblasts and finally these cells were all included within the vessel by a process which, as stated above, I was not able to follow definitely.

After closely studying these early vessels in a large number of living yolk-sacs, the observer is able to establish very clearly the actual relationship between the vascular endothelial cells and the erythroblast or early blood corpuscles. The corpuscles on the yolk are always of a distinctly different shape and size, and lie, as described below, in small groups with originally no endothelial cells around them. The groups are later either surrounded by endothelium or taken into the early vessels as already indicated. Nothing has been observed during a long study of these cells on the living-yolk sac of normal embryos with a free circulation, or on the yolk-sacs of embryos experimentally prevented from establishing a circulation, or finally in sections of embryos of various ages, that would indicate even a tendency of endothelial cells to change into any type of blood corpuscle. The endothelial cell, whether in the free and wandering mesenchymal state or constituting a part of the vessel wall, presents a typical shape and clear appearance entirely distinct from the wandering erythroblasts.

In observing the early yolk vessels certain things may be seen which are most important in interpreting sections supposedly showing the transition of vascular endothelial cells into erythroblasts or primitive blood cells. Frequently, one or more corpuscles become entangled within the spaces and filaments existing between the cells of the vessel wall. Other corpuscles flowing in the current strike these entangled ones and beat against them sometimes for hours before they become disentangled from the vascular pits and holes, to flow again in the current. This is an extremely common sight during the early hours of the circulation of blood in any of the yolk vessels.

It may now readily be imagined that if the embryo was killed and fixed while the corpuscles were tangled in the spaces of the vascular endothelium, a study of sections might produce the impression that the cells of the vessel wall were "protruding into the lumen and assuming the typical characters of primitive blood cells," according to the description of many that imagine the occurrence of such things. I have previously offered another explanation of these appearances and many phenomena observed on the living yolk-sac bear out the point of view. It may sometimes happen when the vascular endothelium encloses a group of primitive corpuscles that one or more of these future blood corpuscles come to lie in the plane of the vessel wall, or may actually seem to form one of the cellular components of the wall.

When the circulation begins, however, this cell becomes loosened away from the wall for mechanical reasons, the lack of long processes, etc., and projects into the lumen to be finally washed away. Any one may readily observe such occurrences who will study the living yolk-sac of Fundulus with a high power microscope and a strong condenser so as to use a darkened field.

All of these observations lead one to conclude that the only connection between vascular endothelium and primitive blood cells is one of association. The endothelial cells never metamorphose into blood cells. It is important here to recall the fact previously emphasized by the author that in those specimens in which there is never a circulation of the blood or plasma the vascular endothelium develops in a perfectly normal manner in the aorta and other intra-embryonic vessels, as well as in the vessels on the yolk-sac, yet in none of these does one find any appearance indicating a tendency of the lining cells of the vessel wall to change into any type of blood cell. Numerous other details from my notes might be enumerated which would bear on this question, but sufficient care has been taken to definitely establish the above crucial points as facts. This may not of course hold for all types of animals but it does for those studied.

I have seen a number of sections on which other investigators have based their claim that vascular endothelial cells do change into primitive blood cells and although inclined towards the acceptance of such a view from a mere acquaintance with the literature, a study of such material has convinced me that the negative interpretation is equally plausible in all cases.

3. *Blood corpuscles on the yolk-sac of teleost embryos*

In all meroblastic eggs except those of the teleost a great sheet of mesoblast is found extending over the yolk as the so-called peripheral or ventral mesoderm, or subvitelline mesoderm. It is this peripheral mesoderm that gives rise to the blood islands of the yolk-sac in selachians, reptiles, birds and mammals The teleost, however, presents a unique case in that the ventral mesoderm does not spread out over the yolk but is included within the embryonic body. The differentiation of this mesoderm within the embryo is much the same as that of the peripheral ventral mesoderm in the yolk-sac of other groups. Thus in the bony-fish the great bulk of blood formation takes place within the so-called intermediate cell mass, the probable homologue of a portion of the yolk-sac mesoderm of other vertebrates.

The intermediate cell mass first described by Oellacher ('73) and later fully studied by Ziegler ('87), Swaen and Brachet ('99, '01), and numerous others, is derived from the primary lateral plate mesoderm, separating away from the median border of this plate. The cell masses of the two sides remain apart in some species and form the future cardinal veins and red blood corpuscles, while in others the two masses unite in the median line to form the conjoined cardinals or stem vein, which is loaded with the primitive erythroblasts—the red blood anlage.

All recent workers on the development of the blood in the bony-fish have considered this intra-embryonic blood formation as being the only source of blood cells in these animals. Several authors, however, Swaen and Brachet among them, have recognized that the cells of the stem vein may become so packed and crowded within the embryo that masses of them are directly pushed out laterally upon the yolk. They have also thought it possible that a very few cells might wander upon the yolk-sac and there form blood, but this has been considered questionable in all cases. No one has recognized the actual occurrence of blood islands upon the teleostean yolk-sac. Even Wenckebach in his study of living embryos, although he made so many important observations on the formation of the periblast and yolk vessels, entirely overlooked the early or primitive yolk-sac blood cells. This is probably due to the fact that he studied only normal embryos. In embryos without a circulation of the blood one observes the yolk islands much more readily as the cells finally become filled with haemoglobin and present a bright red color. When they are once located in these experimental embryos it becomes much easier to trace them in the younger normal individuals, and finally the observer readily locates these cells and may follow completely their migrations and association to form the yolk-sac blood islands.

The arrangement of these wandering cells in the yolk-sac, figures 7, 8 and 10, suggests in a way the regions of growth of the yolk-sac mesoderm in other meroblastic eggs. About the caudal region there is an 'area opaca' formed by the great number of wandering cells, while around the head end the scarcity of mesenchymal cells might be considered an area pallucida.

All of the yolk-sac blood islands in the Fundulus embryos are formed from certain of the early wandering mesenchymal cells on the yolk. During the early wandering stages when the future endothelial cells have a spindle shape and the future chromatophores are large amoeboid cells, other mesenchymal cells on the yolk are small and more or less circular in outline. These small circular cells move slower than the other types and throw out short thick pseudopod-like processes.

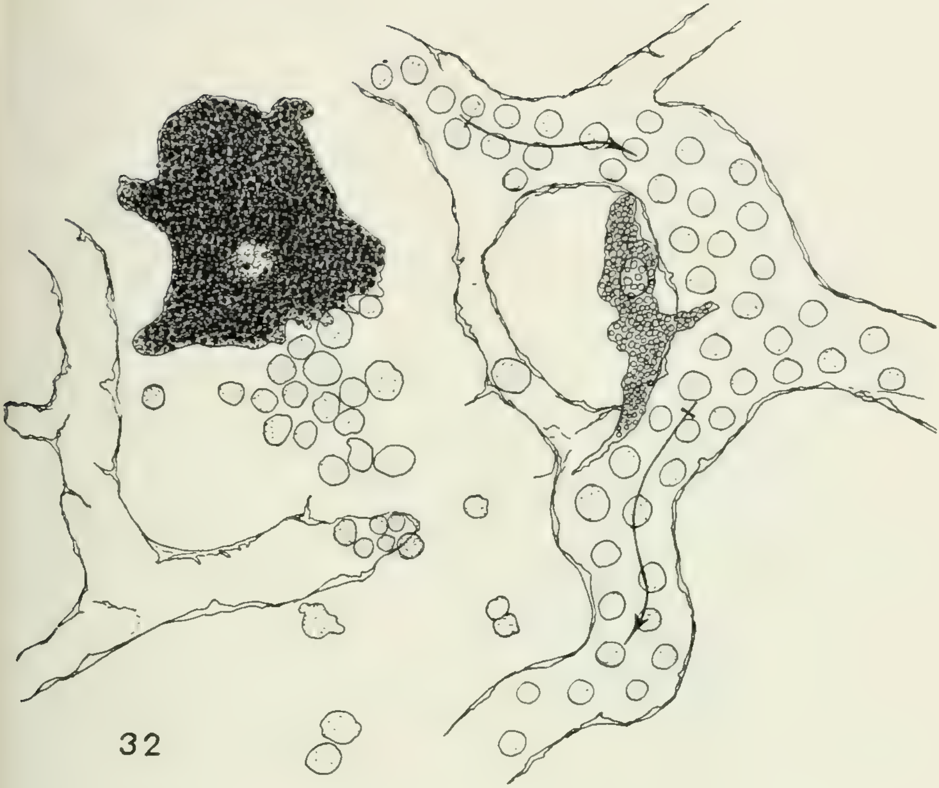
Whereas the spindle shape cells wander away from the embryo along its entire lateral border as well as the caudal end, and the large amoeboid future chromatophores have almost an equally extensive place of origin, the small round cells wander out only from a limited region. The earliest ones of these to be seen are near the caudal end of the embryo before the tail fold has completely separated the caudal end from the yolk surface. As the tail is moulded free from the yolk-sac, the point of outwandering of the circular cells follows the place of union between the ventral wall of the tail and the yolk-sac. Just at this place the mesenchyme of the embryonic body extends itself out on to the yolk as free wandering cells.

In a study of sections this mesenchyme is found to lead directly to the end-bud, Endknospe, which may be considered to represent the blastopore lip. The cord of mesoblast which has been designated as intermediate cell mass leads caudally to the end-bud which is well out in the tail. The ventral cells from this mass wander away into the yolk-sac from the extreme caudal position and other cells also wander away laterally from the intermediate cell mass. Figure 8, the tail end of an embryo 56 hours old, illustrates very well the place of outwandering of the round cells. Few, if any such cells wander out from the lateral borders of the embryo in regions more cephalad than this.

This confined local origin of the round cells and the period at which they wander out, along with their general appearance, lead one to believe that these cells are actually derived from the same general mass or group of cells which goes to foim the intermediate cell mass or red blood anlage within the embryo. All of these slowly wandering circular cells finally differentiate into red blood corpuscles, as described below, just as cells of the intermediate cell mass will finally do.

In this connection a most instructive defect is frequently found among embryos developing in the stronger solutions of alcohol. Many such embryos are of the common short type with their tails split, cauda-bifida, figure 4 of the previous paper. This defect is due to the fact that the germ-ring descends over the yolk in a slow or arrested fashion and may never succeed in fully enclosing it. The caudal end of the embryo is thus divided and the two tail moieties remain spread apart laterally along the line of the germ-ring. This condition renders the caudal portion incapable of including all of its usual median tissues and such cells extend past the angle of the split and lie between the two parts of the bifid tail. The interesting thing is that the cells constituting the blood-forming intermediate cell mass lie in just this position.

Figure 33, a diagram, illustrates the embryonic body with a bifid caudal end. The great lake of blood corpuscles is situated beyond the angle of the split tail. Such an abnormality liberates the future blood forming mass from the body of the embryo and the mass spreads posteriorly over the yolk surface, yet not in a diffuse manner since it maintains its densely packed cellular structure. We might consider that here the evolutionary events are reversed. The blood anlage in the primitive fishes, the selachians, is spread over the yolk in the area opaca. In the normal teleost this primary yolk-sac blood anlage has been included within the embryonic body and localized in the intermediate cell mass. While in the abnormality here considered the intermediate cell mass is again outspread upon the yolk somewhat suggestive of the old ancestral selachian arrangement. This abnormality, in other words, may give some notion of the actual



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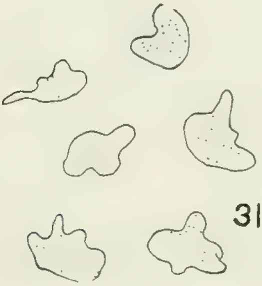

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Fig. 31 A group of six early erythroblasts unsurrounded by endothelium on the yolk-sac of a 90 hour embryo. Short amoeboid processes project and the cells move very slowly.

Fig. 32 A camera lucida sketch of a microscopic field on the yolk-sac of a 90 hour embryo. All four derivatives of the wandering mesenchymal cells are shown. The circulation is established in the vessel to the right and the current follows the direction of the arrows. To the left is a small vessel not yet connected with the current; its wandering endothelial tip is approaching a group of erythroblasts still uninclosed by endothelium as they lie near the huge black chromatophore. A brown chromatophore is seen on the large vessel.

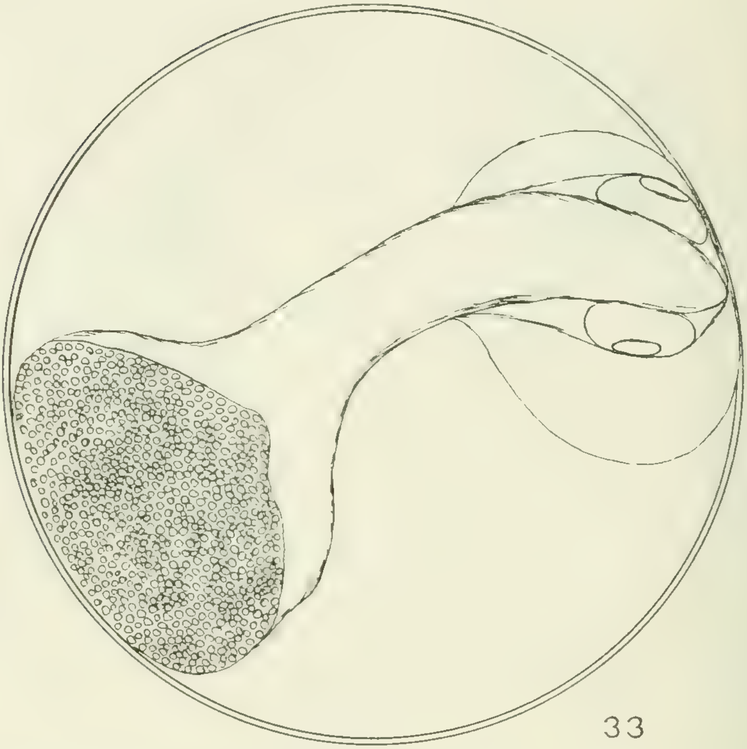

Fig. 33 An outline of a short 6 day embryo from a strong alcohol solution. The embryo presents a cauda-bifid condition and the normally intra-embryonic blood-forming mass is represented by a densely packed expanse of corpuscles outside the body of the embryo and spread upon the yolk.

incorporation of the primitive blood-forming mesoderm of the yolk-sac into the body of the teleost embryo.

The situation may be pictured as follows. In the reptiles and birds, for example, the peripheral mesoderm is outspread over the yolk and in it differentiates the blood islands of the area vasculosa. The peripheral mesoderm in Fundulus and teleosts generally does not become outspread over the yolk, but is concentrated into a median cord or mass within the caudal half of the embryo. Yet even here there is actually a tendency for the cells of this mass to be attracted to the regions of the yolk-sac, and during the early stages of development many cells separate from the mass and wander freely on the yolk. The extent of such wandering is probably variable in different species. Yet in all eggs with an extensive vitelline circulation the wandering of these future red blood cells probably takes place to a considerable extent in spite of the fact that the cells have been so generally overlooked. A fact easily accounted for when one realizes that most of the studies in blood origin in teleosts have been confined to sections of the embryos of the salmon and trout. Sections are extremely slow in revealing the significance or even existence of wandering cells in development.

We may now consider the individual wandering cells and their subsequent differentiation. Figure 31 shows a group of six such cells. They are small when compared with the huge chromatophores but are about as large as the endothelial cells, though completely different in shape, texture and behavior. Figure 32, a camera lucida sketch, serves well to illustrate the relative sizes of the different type cells on the yolk-sac. In this figure are shown all four types, the enormous black chromatophore, the very large brown chromatophore, the delicate elongate endothelial cells of the vascular wall with their filamentous processes, and the almost circular erythroblast, small when compared with the first two types, but as large or even larger than the endothelial cell.

Figure 34 was drawn from an embryo that had been fixed in such a way as to render conspicuous the cell outlines of the ectodermal layer of the yolk-sac. Below the ectoderm groups of erythroblasts forming blood islands are shown, and the extremely small size of the erythroblasts in comparison with the enormous dimensions of the ectodermal cells is most striking.

As mentioned before, the erythroblasts tend towards a circular shape but send out short processes, while they move in a sluggish amoeboid fashion. The cytoplasm of these cells is slightly greyish and not so perfectly transparent as that of the spindle cells; this difference between the two types is not so marked during the early stages but is readily noticeable in embryos of 80 or 90 hours.

The future erythroblasts very slowly wander away from the tail region of the embryo and down the posterior surface of the

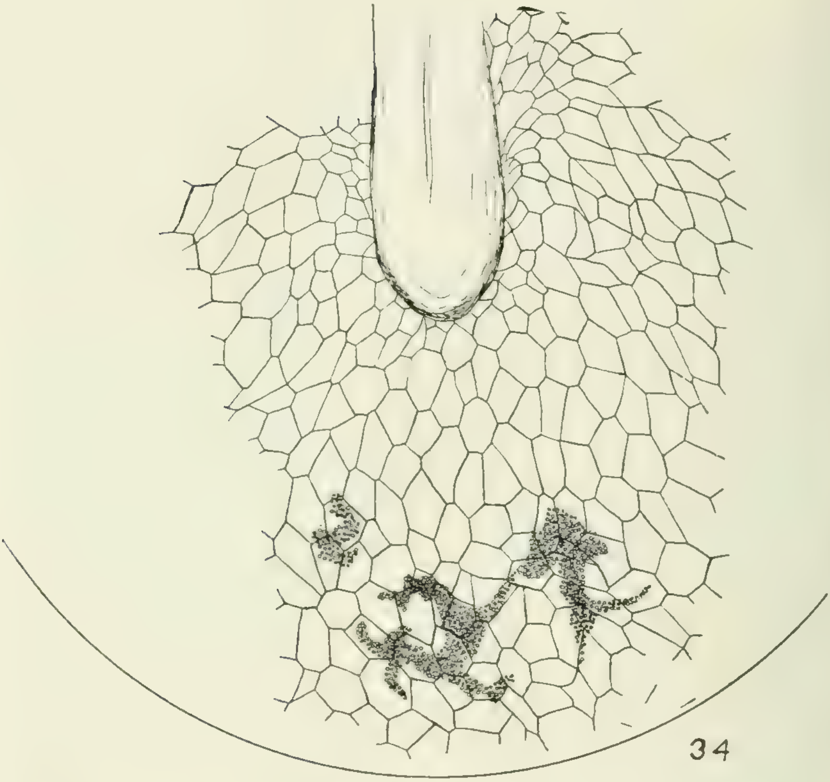

Fig. 34 A camera lucida outline of the caudal region of the yolk-sac in a 96 hour embryo with the ectodermal cell borders made visible by mercury fixation. An island of blood corpuscles is indicated by small circles which give some idea of the minute size of the erythrocytes as compared with the huge ectodermal cells.

yolk to reach its ventral surface. At various places on the posterior and ventral yolk surfaces the cells collect into groups and become less active, although their movement does not entirely cease. These groups constitute early blood islands. They are at first not surrounded by endothelial cells but later become enclosed or taken into the ends of the incipient vessels as briefly described above.

When the circulation first begins on the yolk-sac of a normal embryo a great many of these islands are present, but are more or less isolated or out of the channels through which the fluid is flowing. The taking up of the islands by the circulation is most interesting to observe. At first those cells near the tail of the embryo, which are enclosed by endothelium, are taken. A few of the corpuscles are shaken by the current and these strike against the other members of the group until all become loosened and move slightly to and fro; finally one or two are suddenly washed away, then others follow few at first, until the entire group loses its stand and is swept away by the current. One and then another of the islands may be seen to perish in this manner before the irresistible force of the tiny stream.

Yet even after the yolk circulation is fairly well established a number of islands of the round cells may still exist unsurrounded by the endothelial vessels. Figure 32 shows such a case. A well established current flows through the vessel to the right, while to the left is an incipient vessel not yet connected with the current. In the center of the figure is a group of corpuscles below a huge black chromatophore. These round cells constitute a blood island still unenclosed by vessel endothelium; in the course of a few hours, however, they too will become enclosed by a vessel and subsequently be included among the circulating blood cells.

The early erythroblasts which are in this manner included within the circulation assume a circular outline as they float in the current. In the previous paper, figures 31 and 32 of cross sections through the intermediate cell mass, and figures 34 and 35 of cells in a yolk island, all from a young 72 hour embryo, illustrate the definite circular outline of these cells. In life they may be carefully observed in the small vessels where a single cell passes with difficulty, and are here seen to be globular in shape and to retain their slight amoeboid movement. The form of the early erythroblast is readily changeable for the first one or two days after entering the current. After two or three days, that is, in embryos five or six days old, the cells in the blood current assume a typical erythrocyte appearance, becoming elongate and elliptical in shape when seen from one position, while they are thin in profile view. They are now ellipsoidal nucleated red blood corpuscles. At about the time they begin to change from the globular to the elipsoidal shape, the accumulation of haemoglobin takes place and the cells begin to show a typical straw color.

It may then actually be seen in the living that the early or primitive erythroblast is really a more or less globular amoeboid cell without haemoglobin and resembling more closely a lymphocyte or early leucocyte than a fully formed erythrocyte. This is probably true of the early stages in many cases of cytomorphosis, yet the globular amoeboid cells of the yolk islands are not indifferent 'primitive blood cells' in the sense Maximow ('09) has concluded, but they are definitely future erythrocytes.

This point is established by a study of these cell groups in the normal as well as in embryos without a circulation of the blood. In the latter individuals the islands arise by the formation of local aggregations of the early wandering cells in an exactly similar manner to that described for the normal embryo. In fact, the observer cannot distinguish between the two specimens in many cases, yet one fails to establish a circulation and the islands are thus enabled to retain their positions on the yolk-sac.

The constituent cells of such a permanent island may be observed from time to time or continuously, and will be found to pass through changes exactly identical with those which take place in the island cells that become swept into the blood stream of the normal embryo. They are for a few days globular in shape, but capable of slightly changing their form and sending out short pseudopod-like projections. When the embryos are five or six days old the cells in these islands then become flattened ellipsoidal corpuscles and attain a haemoglobin content exactly as in the normal embryo. The blood islands now appear bright red in color and are quite conspicuous on the yolk-sac where they permanently remain. The globular colorless cells are thus seen to differentiate directly into the typical ictheoid erythrocyte.

From a study of the living embryos alone one could not, of course, be certain that all of the cells of these islands had differentiated into red blood corpuscles. However in the previous study of the non-circulating embryos, I have examined a large number of yolk islands completely and have never seen any type of blood cells other than erythrocytes in such a position. The same is true of the cellular products of the intermediate cell mass; in hundreds of sections studied with the oil immersion not one case has been found of a lymphocyte or leucocyte arising from a cell of the original intermediate cell mass. It is thus concluded that this mass is the red-blood anlage of the teleost and the wandering cells of the yolk-sac which are very probably derived from the same mesodermal stem that forms the intermediate cell mass are likewise a portion of the red blood cell anlage.

The embryos that fail to develop a circulation are most important material for the study of these questions of relationship among the different types of blood cells. The observations on the living show definitely the qualities of the early blood forming mesenchymal cell in its assumption of the globular slowly wandering type, which would fully satisfy the descriptions of Maximow's 'primitive blood cell' as being closer in appearance to a lymphocyte than to a red corpuscle. When one follows these supposedly indifferent primitive blood cells which are claimed to possess the power of differentiating into either a leucocyte or an erythroblast, he invariably observes them to differentiate only into erythrocytes. Although all cells of the early islands are distinctly visible, they are not mixed in type, but are of one class. A long study of these early islands in sections also fails to reveal any other than red blood cells. The leucocytes are not very numerous in the blood, yet they should be seen if present in these islands, as they are found in other positions and are well represented in the blood of the adult Fundulus.

The further history of the cells in the stagnant masses on the yolk-sac of an embryo in which the blood has never circulated is instructive in several ways. In the first place, all of these islands seem to become surrounded by endothelium, yet the endothelial arrangement cannot be completely traced in every case and it rarely extends much beyond the island mass so far as one can observe in life.

Pigment cells are irregularly scattered in the neighborhood of the islands, as they are throughout the yolk region. The chromatophores do not, however, assume any arrangement with reference to the islands of cells and as mentioned above they retain their original condition as separate cells instead of fusing into syncytial masses, as is the case in specimens with a free blood circulation.

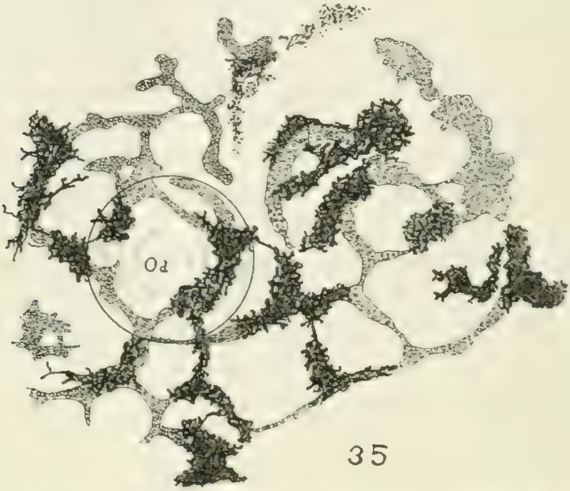

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Fig. 35 The arrangement of red blood corpuscles in more or less connected endothelial sacs on the ventro-lateral surface of the yolk in an embryo 14 days old that had never had a circulation of fluid in its vessels. All of these blood cells wandered away from the caudal body regions of the embryo during early stages. The chromatophores are irregularly scattered among the old blood-islands. *Od*, oil drop.

Figure 35 illustrates a group of blood masses on the yolk-sac of an embryo 14 days old. In this specimen there had been absolutely no circulation or movement of the blood fluids within the vessels *at any time.* This is most important to know in the case of all specimens without a circulation at the period they chance to be examined. I shall return to this point below. The cell groups in the specimen without a circulation are arranged somewhat like a vascular net in the region illustrated by figure 35, yet they present a deadly still appearance as contrasted with the lively movement of the corpuscles within the yolk vessels of a normal individual. The erythrocytes forming these islands are brilliantly red in color and their shape and size are apparently normal.

The blood corpuscles are thus found to differentiate in a typical fashion and to retain their haemoglobin reaction for a long period without having circulated in the vessels. The function of an erythrocyte would thus seem to be entirely independent of its circulation so far as its capacity to form oxyhaemoglobin goes. These cells are also able to accumulate oxygen within the intermediate cell mass in its central position in the embryonic body. The embryo from which figure 35 was taken had lived 14 days without its blood having circulated, which is about the period required for the young fish to hatch and become free swimming.

In older embryos the erythrocytes begin to degenerate and in many they lose their red color, the haemoglobin probably breaking down. The islands on the yolk then become pale in color and finally almost white, as if the cells were dead. The color of the blood cells seems to fade within the embryo earlier than on the yolk-sac as a rule, probably due to the better chances of obtaining oxygen on the thin yolk-sac than in the thicker embryonic body. The non-circulating specimens often continue to live for a long time even after the blood cells have lost their color. Some such specimens may exist for more than 40 days, which is a very long time considering that the normal embryo may hatch when from 11 to 20 days old. The specimens without a circulation are always weak and delayed in development and of course never succeed in hatching from the egg membrane.

In a study of the embryos treated with weak alcohol solutions, one very frequently finds cases in which the circulation of the blood may start almost normally and finally stop permanently, although the embryo continues to live. Other embryos may fail to establish a circulation at the proper time and yet may develop a freely flowing circulation of their blood some days later. Again, an embryo may have a fairly normal circulation and for some reason lose it for a few hours, or for one or two days, and then regain it, at first slowly and finally in a fairly strong fashion.

All three of these phenomena have also been observed in eggs developing in ordinary sea-water when they were not properly separated so as to allow free respiration. The egg membrane is covered with long hair-like filaments which become entangled with those of neighboring eggs and in this way masses become closely packed. The central eggs of such a mass develop in a poor atmosphere and go more slowly than their neighbors on the outside of the mass. These centrally located eggs show many abnormal and arrested conditions of much the same type as may be obtained by treating the eggs with various injurious solutions.

The changeable states in the circulation offer many pitfalls for one attempting to determine the sites of origin of blood cells in the non-circulating embryos. Old embryos are seen in which there are beautiful blood islands on the yolk-sac and great clots of blood in the head or other unusual position. The heart is very frequently completely loaded with corpuscles, and yet there is not the slightest movement of the blood cells or any sign of a circulation at this time. The heart itself may be pulsating feebly or even practically stopped.

Another source of blood movement which is slight, yet to be guarded against, is that due to the muscular twitching of the embryo's body. This movement may frequently serve to push cells from the intermediate cell mass out on to the yolk-sac, but usually by way of the vessels. These dangers are to be taken seriously in experiments of this kind. Since one is able to be *absolutely certain* that the blood never circulates in a great number of embryos, only such embryos should be considered in a study of blood origin. During a study of this exact problem now extending over four spawning seasons, I have seen blood in almost every conceivable position in embryos without a circulation at the time of the observation. The accumulation of blood is more frequent in certain positions and regions than in others. The venous end of the heart is a most common place for a clot, the sides of the head, the large vessels of the yolk just lateral to the body, and various places on the anterior and lateral yolk surfaces.

When, however, the experimenter collects a number of embryos that have really never experienced the slightest flow of their blood, the case is very definite. No blood clots ever occur in regions other *than the 'intermediate cell mass,' within the embryo, and the islands on the caudal and ventral yolk surfaces which have been formed as described by the early wandering cells that migrate away from the caudal region of the embryo. All embryos whose history for lack of circulation throughout their existence is actually known show the blood pattern most consistently, there being of course a certain amount of variation in the extent and position of the yolk-islands but not enough in any case to confuse the problem.*

These observations may readily be made by any observer, but can only be made in a reliable fashion with the high power microscope and strong condensers so that the light may be sufficiently regulated to observe the most transparent cells. The movements and differentiation of these cells should be carefully followed through every step in a number of cases, in order to fully appreciate the significance of their position and behavior.

The cells may be seen even with an ordinary binocular microscope to some extent, but the arrangements for light regulation and the magnification are insufficient for determining the important details. After the red blood cells have formed, they are readily located even with a low power yet such an examination could only determine their places of origin provided the embryo has been carefully watched with a high power magnification to make certain that it has had no blood flow.

The condition of the yolk-sac mesenchyme must be fully understood and must always be considered in interpreting the origin of blood-islands and clots. For example, clots seen at the venous end of the heart or on the extreme anterior surface of the yolk must be most cautiously considered, remembering the scarcity or even absence of the wandering mesenchyme in these regions. Clots in such places probably always result from a partial circulation of short duration and there is abundant evidence to support such a view.

Although the future red blood cells migrate upon the yolk in their early mesenchymal stages, after they once group themselves and differentiate into erythrocytes their powers of wandering become very much limited if they exist at all. I have never seen anything to indicate that a fully formed erythrocyte was capable of automatic migration. Yolk-sac blood islands of all ages have been examined in great numbers, but never has an erythrocyte appeared wandering away from such an island into neighboring regions. This fact is most important in the study of the blood-islands in the non-circulating specimens.

When the slightest flow does exist for any length of time, there is a definite tendency, as mentioned above, for the blood to accumulate in certain sinuses and vessels. The positions of accumulation vary somewhat with the stages at which the circulation ceased, as well as the manner of stoppage of the flow, whether it was gradual or sudden.

When the circulation stops during early stages, there is a great accumulation or massing of the blood over almost the entire ventral surface of the yolk. In other words, there is a hemorrhage or bleeding into these spaces or vessels until no more blood is left in other regions of the embryo, the heart gradually becomes empty of corpuscles and no longer passes them along. The packing of the yolk vessels probably clogs or blocks the circulation so that it ceases. Again, the circulation may stop more suddenly and the venous end of the heart or the entire heart may be seen packed with corpuscles while the vessels immediately entering and leaving it are comparatively or entirely empty. In older embryos there is the tendency to accumulate red cells in the vessels of the head so that brilliant red clots are frequently seen in these positions.

In all cases it is interesting in these individuals in which the circulation has ceased at one or another period in development, and doubtless for different reasons in different specimens, to observe the way in which the blood sooner or later accumulates in one or another vascular space and does not remain uniformly distributed throughout the vascular system. Only when the heart is suddenly stopped and the blood quickly fixed by some strong killing fluid does one get a good pattern of the vascular system loaded with corpuscles throughout most of its extent. In rare cases, three during the present summer in some hundreds of embryos examined, will a specimen without a circulation at the time observed show almost all of its vessels loaded with blood cells, and this is probably due to a slowing down gradually of the circulation on account of the heart itself which finally stops with the vessels in a balanced state.

The study of the yolk-sac in the living embryo enables one to observe every phase in the development of the red blood corpuscles from the early time when they wander as amoeboid mesenchyme cells to collect into groups of globular cells with short processes, the 'primitive blood cells' of descriptive histologists, to be later surrounded by vascular endothelium, and then to change from the globular wandering cells into the flattened ellipsoidal erythrocyte loaded with haemoglobin, and finally freely floating in the current of the blood stream. The fully formed corpuscles apparently become incapable of independently migrating even when not carried by the circulation.

DISCUSSION AND CONCLUSIONS

In the previous paper on the origin of blood and endothelium, a somewhat full discussion of the problems of blood formation in the teleosts and other vertebrates was entered into. A consideration of the questions of origin and development of vascular endothelium was also undertaken in the light of the results there presented and the more recent general literature bearing on this subject. The experimental results then contributed seemed in the light of the past literature to render highly probable, if not to actually prove, the polyphyletic origin of the various types of blood cells, as opposed to the now extremely improbable monophyletic theory of origin of blood cells and vascular endothelium. For a general consideration, the reader is referred back to these discussions.

A number of particularly significant points are brought out in the present study of the living normal and experimental embryos which bear directly on several of the past theories and speculations regarding the origin of vessels and blood. Only these special points will be briefly considered and analyzed at this time.

In the first place, the writer cannot resist the impulse to highly recommend that all students of haematogenesis and vascular origin spend some time at least in a study of living mesenchymal cells and their cytomorphosis. Such a study will soon convince one of the great disadvantages under which an investigator labors in attempting to solve the origin of blood from observations on dead material in serial sections. The problem becomes so simplified and devoid of laborious uninstructive technique that it seems almost superficial. One may learn as much from the living yolk-sac in an hour of careful study as in almost a week's perusal of sections. Most important is the fact that certain things may actually be seen to occur that sections could scarcely stimulate the mind to imagine. The only disadvantage is that the worker may be led to wonder whether so apparently simple a problem is actually of scientific importance. Fortunately this mental state is soon passed over on realizing the necessary care and precaution which must be taken in following the movement and changes in the living cells.

Each cell must be recognized as a living complex and the observer will realize the importance as well as the difficulties of thoroughly understanding and interpreting correctly its manifold changes and behavior. Material which to some extent allows such a study is often available. The Fundulus yolk-sac, however, is exceptionally adapted to this study on account of the beautiful simplicity of its structure, as well as the remarkable clearness with which each cell may be observed.

An investigation of the Fundulus yolk-sac readily supplies a crucial answer to the old question regarding the relation of the blood vessel lumen to other body cavities and spaces. Ryder ('84) was right in describing the blastocoel of the bony-fish as remaining an extensive cavity for some time. This is the space between the ectoderm and yolk and is identical and continuous with the cavity which arises very early beneath the blastoderm and above the yolk periblast. Agassiz and Whitman ('84), as well as Ryder ('84), Wilson ('90), and others, have identified this correctly as the cleavage cavity, the blastocoel. Later in development, the blastocoel extends over the yolk, forming the space into which we have seen the free mesenchyme cells wander, and finally within this space groups of these cells form the yolk-sac vessels.

Wenckebach ('86) described very clearly the origin of vessels from the free mesenchyme within the segmentation cavity. My study of a somewhat similar yolk-sac confirms the main points brought out by Wenckebach and all serve as crucial facts in support of the early theory advanced by Bütschli ('82) in his "Die phylogenetischen Herleitung des Blutgefässapparates der Metazoen." Bütschli held that in the Metazoa the lumen of the blood vascular system was derived from the blastocoel. Later, Hubrecht ('86) supported the same standpoint from his studies on Nemertines. Hubrecht also found wandering cells playing an important rôle. Ziegler ('87) gives a most careful analysis of the continuity of the vascular lumen with the blastocoel in his studies on the development of the bony-fish.

The foregoing description and figures of the origin of vessels on the yolk-sac of Fundulus leaves no doubt that the vascular lumen in these animals, coenogenetically at any rate, is continuous with the blastocoel or primary body cavity and is in no way related to the coelom.

Almost twenty years ago, Felix ('97) advanced the opposing theory that the vascular lumen was really a localized or separate part of the secondary body cavity, or true coelom. The many decided objections to this theory from the standpoint of comparative anatomy, the presence of blood vessels before the acquisition of a true coelom in the animal kindgom, and the numerous embryological contradictions in its path were pointed out in the discussion of this matter in the previous paper.

Very recently Bremer ('14) has advocated the theory of the origin of vessels as parts separated from the coelomic cavity, or strands of cells from the coelomic epithelium. In the first place, the material on which his investigation was based, early human embryos, will scarcely permit such generalizations. At least more suitable material could be found for the analysis of this problem. Further than this, his consideration of the questions involved does not lead one to form a definite idea of the exact direction he considers his evidence to lead. He credits Bütschli ('82) with having originated the coelom theory that accords, so he thinks, with his evidence. This is entirely incorrect, at Bütschli's theory is exactly on the other side. The morphology of the yolk-sac of the chick, sheep and numerous other animals, as the literature of the subject readily shows, is entirely out of accord with such speculations. The yolk-sac of the bony-fish shows this view to be really impossible and there should be no longer any doubt that vessels arise from loose and wandering mesenchymal cells in many animal species, and certainly not from ingrowths from the coelomic epithelium in any species.

The formation of vessels on the yolk-sac of the teleost further limits the generalization of the origin of larger vessels from capillary nets. Thoma ('93) in his masterly study of the vasculogenesis of the yolk-sac of the bird, held that "the first vascular spaces, the rudimentary capillaries, were formed by the secretory activity of the cells forming their wall." These capillaries formed an extensive net and the arteries and veins arose secondarily and differentiated from the capillaries on account of the flow of blood set in motion by the beat of the heart. The anlage of the vascular system was the capillary.

These principles of Thoma are not, however, applicable to the development of vessels in the embryonic bony-fish. The aorta arises as one or two vessels independent of any flow of blood or the existence of a capillary net. The first vessels on the teleostean yolk-sac are the large vitelline veins, as described by Wenckebach, and the median vitelline vein or the net of vessels in its place. These large important channels arise entirely independently and separated from the capillary net if such exists at the time. They also develop entirely independently of the blood flow, and not as a result of the pressure due to the heart beat. The capillaries and other vessels in many cases arise separately or away from these primary vessels and finally connect with them in a way similar to the connection formed between the Randvene and the venous end of the heart. Other capillaries and small vessels arise as buds or sprouts from the first formed veins on the yolk-sac.

More recently Evans ('09) with a very efficient and delicate method of injection has shown many of the larger intra-embryonic vessels of the chick embryo to develop from a foregoing capillary network. He found the same principles of development that Thoma had observed on the yolk-sac to hold for the development of certain vessels within the embryo. These principles of vascular development Evans thought applied to vertebrates generally, but such is certainly not the case, the large vessels of the teleost embryo arise directly from associated mesenchymal cells and are not preceded by a capillary net.

His evidence was derived from injected vessels and could not justify the statement, p. 512, of "The presence *always* in the embryo of a united vascular system"—"a single branched endothelial tree." Such a united vascular system is rather late in its establishment in the fish embryo and there is no "single branched endothelial tree" present when the first blood vessels are formed. These facts may readily be demonstrated on the living embryo by direct observation.

The vessels of the yolk-sac and several of the larger vessels within the body of the teleostean embryo form independently of any foregoing capillary network. *In the teleost, then, the anlage of the vascular system is not the capillary but the mesenchymal cells which directly give rise to the chief arteries and veins, as well as to numerous groups of isolated capillaries.* Other small vessels and capillaries grow as branches or sprouts from the arteries and veins.

Thoma advanced three laws for the formation and growth of vessels. The first law was considered the most important, but rather destructive evidence is thrown against it by the present study. The law may be stated as follows: "The increase in the size of the lumen of the vessel, or what is the same thing, the increase in the surface of the vessel wall, depends upon the rate of the blood current." The vessel increases in size when the rate is exceeded, becomes smaller when the rate is slowed, and *disappears when the flow is finally arrested.* Thoma ('96) states: "This law which brings the growth of the surface of the vessel into dependence upon the rate of the flow of the blood is, I consider, the first and most important histo-mechanical principle which determines the state of the lumen of the vessel under physiological and pathological conditions."

Thoma again states this principle thus: "In development the vessels in which the blood stagnates degenerate, and in those in which the rapidity is too great the lumen in enlarged."

No one could fail to admire the splendid manner in which Thoma attacked the problem of vasculogenesis in the yolk-sac of the bird, or the ingenious way in which he attempted to analyze the problem and deduce his three laws of histo-mechanical processes. Yet the "First and most important histo-mechanical principle" does not apply to the development of vessels in Fundulus embryos where there is no circulation of the blood. *Many vessels grow in size or "what is the same thing, show an increase in the surface of the vessel wall" without any "rate of the blood current."* The aorta in old embryos that never had their blood to circulate and in which the heart is actually a solid string of tissue, grows and attains a well developed lumen and a wall lined with endothelium and surrounded by concentric fibers of connective tissue as is shown in figure 49 in the previous paper, drawn from such a specimen. This vessel is very slow to degenerate, in fact, it shows no sign of degeneration and actually persists as long as the embryo is able to exist without a circulation, for 30 days or more. Vessels also develop upon the yolk-sac without ever having a fluid to circulate through their lumen. *Other vessels are developed around the blood cells of the intermediate cell mass and the yolk-sac islands and in such vessels 'the blood stagnates' from the first yet the vessels degenerate very slowly, in some cases scarcely at all.*

In still other cases the blood may have circulated for a while and then stopped for some time, but the vessels do not degenerate as is proven by the fact that the circulation through them may again be resumed. Such a sequence of events may be occasionally observed in the experimental embryos. *The function of the vessel as a blood conductor, therefore, seems in these embryos of Fundulus,* both the early normal and those without a circulation of the blood, *to have little if anything to do with its early development and not much effect on its ability to survive.*

On the other hand, when Thoma has a straight normal case, the lumen may readily be seen to increase in size with the rate of flow. Yet in the entire absence of this action the vessel is still capable of increasing in size and it becomes questionable whether the rate of flow is ever an actual cause of size increase beyond mechanical stretching.

These facts are most significant in a consideration of the influence of function on growth and development, auto-differentiation. Here it is seen that the structure both grows and develops in entire absence of its function. In normal cases the function of the vessel as a blood conductor exerts more likely a physical rather than a biological effect on development.

Thus the development of blood vessels on the yolk-sac of the living Fundulus embryo proves that the capillaries are not universally the anlage of the arteries and veins, but that these larger vessels may arise directly from wandering mesenchyme cells. Such arteries and veins may grow and persist without a circulation of the blood through their lumen and even though stagnant masses of corpuscles may crowd the vessel cavity.

The development of vessels in Fundulus also directly disproves the claims made by Sobotta ('02) that the vessels in the teleost grow over the yolk entirely as branches from those near the embryo and without the wandering cells taking part. This assumption is probably due to the difficulty of estimating the part played by wandering cells from a study of serial sections. From a study of the living yolk-sac there is no question of the major part played by the wandering cells in the origin and formation of vessels on the yolk.

Sobotta also advances the opinion that the entire yolk vessels may sprout from the heart. This is much of the same nature as the ingrowth or parablast theory of His ('75), and is obviously defeated by the same array of facts which long ago relegated the parablast theory to a place of mere historic interest, in spite of the fact that it is so often revived for literary reasons.

Finally, we may consider the study of the developmental products of the early wandering mesenchymal cells on the yolk-sac of the Fundulus embryo as a problem of cell lineage carried to its ultimate end. The primordial mesoderm cell or cells carry within their bodies all the potentialities of the mesoderm and may give rise to a series of cells which are capable of developing muscle, cartilage, bone, connective tissue proper, blood cells, vessels, etc. Yet after a few cell generations the individuals in the series derived from these early cells containing all the mesodermal potentialities no doubt become somewhat limited as to their potentialities. In a certain generation there may be definite cells more or less generally distributed which possess the capacity to give rise to muscle cells, but to no other type of mesodermal tissues. Still later in development these cells may come to be even more limited in their developmental capacities and thus have the power to produce only a certain type of muscle cell and no other type.

Collections of such cells would then be designated embryologically as the anlage of striated muscle, smooth muscle, or heart muscle as the case might be Yet it is not to be forgotten that at this stage there might be really no means of distinguishing between the several different types of mesodermal cells.

Limitization of potentialities in the individual cells has apparently reached a comparable stage just about the time when the mesenchymal cells begin to wander upon the yolk-sac of Fundulus. We have seen these cells as they wander out and have noted how very soon they may be separated into four distinctly different types, and following the development and behavior of these types it has seemed evident that they are entirely separate and do not intergrade or transmutate. The black chromatophore does not change its nature or divide off other cells which become different in type from the parent cell. Neither do the endothelial cells lining the vessel walls change into chromatophores or into erythroblasts, or vice versa.

From all the observations on these yolk-sacs we must conclude that the four types of cells described above have developed from four different anlagen, although these anlagen were not necessarily localized groups of cells, but were diffusely scattered mesenchymal cells capable of developing into a definite product, either normal or abnormal, depending upon the nature of the developmental environment. Therefore, the four distinct mesenchymal anlagen each give rise to a perfectly typical and distinct cell type although all develop in, as far as one can judge, an identical environment, the cavity of the yolk-sac between the ectoderm and the periblastic syncytium. The differences among the four cell types produced are from the standpoint of our present knowledge in all probability due to the potential differences among the apparently similar mesenchymal cells from which they arose. The four types including endothelial cells and erythrocytes we must consider from an embryological standpoint as arising from different mesenchymal anlagen.

SUMMARY

The yolk-sac of the teleost egg is a most beautiful object for observing the movements and migrations of cells in the developing embryo. Such a yolk-sac has only one really definite continuous membranous cell layer, the ectoderm; a true endodermal layer is absent, though a superficial syncytium, the periblast, fuses with the actual yolk surface. The mesodermal layer is represented by numerous separate wandering mesenchymal cells. These freely wandering mesenchymal cells may be clearly observed through the perfectly transparent ectoderm as they move over the surface of the periblast.

The present contribution attempts to give a full account of the movements of the mesenchyme cells and their manner of development and differentiation in the yolk-sac. Observations have been made on the normal embryos from the earliest stages at which the mesenchyme wanders out upon the yolk up to the late embryo in which a complex vitelline circulation is fully established, and all of the products of the yolk mesenchyme completely differentiated. The study has been greatly facilitated by a comparison of the normal embryos with specimens in which the circulation of the blood was experimentally prevented from taking place. In such specimens the cells on the yolk-sac never became confused or contaminated with other cellular elements introduced by the circulating blood. The wandering cells may thus be completely followed through all stages in their isolated position.

The behavior of the migrating cells impresses one with the very important rôle of such elements in the formation of tissues and organs, particularly the blood vessels and certain blood cells. The observer is also struck by the fact that such phenomena are extremely difficult if not actually impossible to interpret from a mere study of dead specimens cut in serial sections. Of course the study of sections greatly aids the observations on the living, and but for the fact of a long acquaintance with the Fundulus yolk-sac in sections, the writer would have found it much more difficult to identify many of the cells in life.

The results of this investigation of wandering mesenchymal cells may be summarized as follows:

1. The wandering cells begin to migrate away from the embryonic shield or line of the embryonic body at an early period, when the embryo is about 40 hours old, the germ ring having almost completely passed over the yolk sphere to enclose its vegetal pole. The cells migrate away chiefly from the caudal end of the embryo, only a few wandering out from the head region. The regions of the yolk-sac thus suggest an area opaca about the tail end and an area pallucida around the neighborhood of the head.

All of the cells wander into the so-called subgerminal cavity, the space Wilson ('90) and others consider a late stage of the segmentation cavity, between the yolk-sac ectoderm and the periblast syncytium.

When the cells first appear they are all closely similar in shape and about the same size. Very soon, however, they begin to exhibit certain differences. Many become elongate spindle cells with delicate filamentous processes, sometimes producing a stellate appearance. Others are more amoeboid in shape with conical pseudopod-like processes which are constantly being thrown out at one place and withdrawn at another. Still a third class of cells appear somewhat later than the other two; these are more circular in outline with short thick pseudopods and are more slowly moving.

The movements of these extremely numerous cells and their changes of position may be readily followed with a high magnification. In embryos of about 60 hours, still some time before the heart begins to beat or the blood to flow, four clearly distinct types of cells may be recognized among these originally similar mesenchymal cells, and the further history of the four types has been completely traced.

2. The amoeboid cells with conical pseudopod-like processes shortly after 60 hours begin to show an accumulation of pigment granules within their cytoplasm. Just at this time they are seen to be of two distinct varieties, one depositing a black and the other a brownish red pigment.

The black chromatophore increases rapidly in size and by the end of the third day becomes an enormous amoeboid body wandering over the yolk. These cells are attracted to the walls of blood vessels and plasma filled spaces, such as the pericardial cavity becomes in specimens without a blood circulation. When the embryo is five days old the chromatophores are abundantly arranged along the walls of the vitelline vessels, but the pigmented cells are distinctly separate. After this time neighboring cells begin to fuse along their adjacent borders and large pigment syncytia are formed which completely surround and ensheath the vessels. A single syncytium is often of considerable extent, as shown in figure 15.

The brown chromatophores have a somewhat different history. They never become so massive as the black, and their processes are more delicate and graceful in appearance. Yet these cells also attain a large size and in embryos of 72 hours are scattered over the entire yolk-surface. After the third day when the blood begins to flow in the yolk vessels, the brown chromatophores likewise become attracted to the vessel wall. These exquisitely branched cells apply themselves to the wall of the vessel and may often completely surround it, as shown in figure 17. This type of chromatophore, however, always maintains its cellular individuality and never fuses with other cells to form a syncytium as is the case with the black type.

The function of the chromatophores on the yolk-sac is most difficult to decide, but one thing is certain, they never become changed into any type of blood cell. The brown chromatophore in early stages may accidentally reach the blood current; it then becomes spherical and may be readily observed for a long time on account of its huge size as compared with the blood cells. It never, however, changes in type.

In specimens without a circulation of the blood both types of chromatophores arise in a normal manner and differentiate normally. Their arrangement along the vessel walls fails to occur and they remain scattered over the yolk or collected about the plasma filled spaces. The heart in such embryos is sheathed with pigment, while the normal heart never has a chromatophore on it.

3. The elongate spindle cells with their delicate filamentous processes are small in comparison with the two chromatophore types. These spindle cells retain in general their original appearance, but their behavior is most important. In embryos of about 48 hours such cells aggregate into certain rather definite groups; later, these groups become more linear in shape and finally these lines of cells arrange themselves so as to form tubular vessels. Several of the larger vessels arise independently upon the yolk, and certain ones of them later become connected with the venous end of the heart, while in all cases capillary nets which also arise independently become connected with the larger vessels. These processes may actually be followed through every step in the living yolk-sac.

The wall of the early vessels is very irregular with spaces existing between the component cells. Corpuscles are often caught in these spaces or entangled in the filamentous processes of the endothelial cells. Such conditions in sections would appear as though the corpuscles actually formed a part of the endothelial wall and might incorrectly be interpreted as endothelial cells changing into blood cells. Nothing has been seen in the living embryos to indicate that an endothelial cell has the power to produce a blood cell or to change into a blood cell of any type, but much has been seen to the contrary.

The generalization particularly made by Thoma ('93) that larger vessels arise from a net-work of capillaries is not true for the large vitelline vessels on the fish yolk-sac. It is also found in the specimens without a circulation of the blood that the vessels arise and increase in size and persist for a long time without ever experiencing any effect of the blood current upon their walls. In many embryos the circulation after having begun may stop for a time and then later be reestablished, the vessels having persisted in a normal condition. Thoma's so-called laws of vessel formation are, therefore, rudely violated by the development of the vascular system in these embryos.

The vessels arising from independent mesenchymal cells in the space of the blastocoel in the teleost yolk-sac entirely overthrow any notion that vessels arise ontogenetically as portions of the coelomic epithelium. The vascular lumen is originally continuous with the primary body cavity, the segmentation cavity, and never with the secondary body cavity or coelomic cavity.

4. The fourth class of cells wander out from the embryonic body somewhat later than the three former types. These are small globular cells with short pseudopod-like processes. They move very slowly, but finally collect into groups on the posterior and ventral regions of the yolk-sphere.

The round cells wander away only from the caudal region of the embryo and probably are derived from the so-called intermediate cell mass which is the anlage of the red blood corpuscles in the fish embryo.

The groups of round cells are slow in their differentiation but just before the circulation of the blood begins, they are seen to be circular erythroblasts. The observer may follow the disappearance of the islands of cells one by one as they are enclosed by the vessels and swept into the circulating stream. About the fifth day these circular erythroblasts become flattened ellipsoidal erythrocytes filled with haemoglobin, the typical red blood corpuscle. The complete change from wandering more or less spherical mesenchymal cells into typical haemoglobin bearing corpuscles may be followed in the living yolk-sac.

In several instances the entire body proper of the embryo failed to develop or else degenerated very early, yet the yolk-sac formed or persisted with numerous blood islands fully differentiated.

The embryos in which there has been no circulation of the blood form the blood islands from the wandering cells on the yolk-sac, and the constituent elements of these islands differentiate perfectly and may maintain their red color for many days. Yet they never leave the locality in which they have differentiated. The fully formed red blood corpuscles have little if any power of migrating. When the observer can be positive that the blood has never circulated, and this requires very consistent watching, the islands of the yolk are always limited to certain regions and never occur so far anteriorly on the ventral surface of the yolk as to reach the venous end of the heart.

5. On the yolk-sac of Fundulus embryos one thus finds four distinctly different products differentiating from the apparently similar wandering mesenchymal cells. The environment in which the four types differentiate is identical as far as is possible to determine, and the only explanation of their various modes of differentiation is that the original mesenchymal cells that wandered out were already of four potentially different classes. These differences in potentiality within the early cells gave rise to the four different directions of cytomorphosis in one and the same environment. The four resulting types of cells are then in an embryological sense derived from different mesenchymal anlagen.

LITERATURE CITED

(Only those titles not given in the previous paper)

Agassiz, L. and Whitman, C. O. 1884 On the development of some pelagic fish eggs. Proc. Amer. Acad. Arts and Sci., xx.

Bremer, J. L. 1914 The earliest blood-vessels in man. Amer. Journ. Anat., 16.

Evans, H. M. 1909 On the development of the aortae, cardinal and umbilical veins and the other blood vessels of vertebrate embryos from capillaries. Anat. Record, 3.

Ryder, J. A. 1884 A contribution to the embryology of osseous fishes. Report U. S. Fish Comm. for 1882, Washington.
1887 On the development of osseous fishes. Report U. S. Fish Comm. for 1885, Washington.

Thoma, R. 1893 Untersuchungen ueber die Histogenese und Histomechanik des Gefässsystems. Enke, Stuttgart.
1896 Text-book of general pathology and pathological anatomy. Translated by Bruce, London.

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A FURTHER ANALYSIS OF THE HEREDITARY TRANSMISSION OF DEGENERACY AND DEFORMITIES BY THE DESCENDANTS OF ALCOHOLIZED MAMMALS

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Introduction.
Material and methods.
Direct effects of the alcohol treatment on the animals.
Influence of the treatment on the descendants of alcoholized animals.
The influence of internal and external factors on the quality of the offspring.
Relative conditions of the male and female descendants from paternal and from maternal alcoholized ancestors.
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Summary and conclusions.
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INTRODUCTION

A LITTLE more than two years ago the senior author (Stockard, '13) recorded in this journal experiments which had then been running for three years and seemed to show a definite injury of the germ cells by treating mammals with the fumes of alcohol. This injury of the male germ cells is of such a nature that an alcoholized male guinea pig almost invariably begets defective offspring even when mated with a vigorous normal female. At that time it was also shown that F_1 animals, the offspring of treated parents, though themselves not treated, had the power to transmit the defective condition to their young, and such F_2 young were equally if not more defective than the immediate offspring of the treated animals.

In 1914 in a short abstract Stockard showed further that the offspring from F_2 individuals were apparently more defective than their parents and were often grossly deformed. One case was recorded of the occurrence of a litter of two F_3 animals, both of which were extremely weak and neurotic, showing a condition suggesting paralysis agitans, and further than this the two animals were typical anophthalmic monsters. The eyes were completely absent, no optic nerve or optic chiasma or visible optic tracts along the tuber cinereum could be found on a careful gross examination of the brain. The two animals were produced by parents (F_2) that had never been treated with alcohol, the four grandparents (F_1) had also not been treated, while the *three great grandfathers* had been alcoholized and the three great grandmothers were normal untreated individuals.

Defective eyes and absence of one eye or both eyes have been frequently met with in the experiment, as well as the peculiar nervous condition, and these symptoms are to be considered indicative of the injury or change induced in the male germ cells by the experimental treatment, which in the above case was transmitted through three generations. No question could remain as to the action on the germ cells, as only male ancestors had been treated; every female of the line was an untreated animal.

This abstract called attention to the fact that there was a tendency for the results to differ in subsequent generations from treated males as compared with the descendants of treated females – not enough data were then present to offer any explanation of these differences and a consideration of them will be undertaken in the present paper.

At that stage of the experiment it was also difficult to offer an exact analysis of the mode of transmission of the defects and the type of injury induced by the alcohol treatment, since the total numbers were not large and the F_2 animals had only a few matings, while further generations had not become available for breeding.

The same experiments have now been continued for more than five years and a number of animals have been used, over 700, which cover the behavior of four generations and supply data of sufficient extent to allow a more thorough analytical consideration of the heredity problem concerned.

Experiments of this nature on mammals are fraught with many difficulties, slowness of breeding, small size of litters, difficulty of handling, etc. Yet such material offers one very great advantage in that the quality of the offspring and generations studied is of such a complex that one is enabled to detect indications of rather slight injuries or changes in the material carriers of heredity which would not become evident on lower forms with less diversity in their methods of behavior and structural appearance. In other words, we take it that such conditions as are spoken of as racial degeneracy in man and mammals are often very difficult or even at times impossible to detect in lower forms.

These conditions are for many reasons thought to be inherited. If so their inheritance must be due to a pathological condition of the material carriers of heredity, the chromosomes, or what not, since they are not normal states and, like diseases, are constantly arising in normal families on account of one or another form of intoxication. Is it possible then to produce such a racial degeneracy artificially by treating only one generation of the animals and by so doing observe a pathological behavior of the carriers of heredity? Arguing from analogy there must be pathological heredity due to diseased or altered chromosomes in the germ cells just as truly as there is a known pathological behavior of every other organ and tissue of the animal body.

It becomes then a problem to study the possible methods of modifying the chromosomes or carriers of the inherited qualities of organisms in order to further analyze their normal physiological behavior; in the same way that experimental embryology has been able to supply so many valuable clues to the normal processes of development.

In the following pages we believe the facts indicate that individual guinea pigs are now living in this experiment that have had the carriers of hereditary qualities, the chromatin, of their germ cells injured for a longer time than four years. And during this time they have given rise to offspring of more or less degenerate or deformed type, and in some cases these offspring have passed this modified chromatin on through three generations, all of which contain pathological chromatin and show somatic defects and deformities as an index of their tainted chromatic ancestry. Modified chromatin has been living in the experiment for more than four years in five different generations of animals as a result of the treatment on the one original, P_1, parent generation.

We have tried to regulate every controllable source of error and there can be no doubt that the conditions are brought about in the way described. Could the degeneracy which is so pronounced have previously existed in the stock? This question has been controlled in the first place by the use of two entirely different stocks from different sources and obtained one and one half years apart, the first in the fall of 1910 and the other in the early winter of 1912. The responses of the two stocks to the experimental treatment have been identical. As a second method of control every animal has been tested by one or more normal matings before being introduced into the experiment, and only those giving normally strong offspring have been used. A further crucial control is the constant mating of normal untreated animals from both stocks under identical cage conditions with the experimental individuals. These animals continue to breed normally until very old, when they gradually become sterile. But none have ever given rise to a defective or deformed individual, and the rate of mortality of the young indicates the average healthy condition found in normal guinea-pig breeding. There is a striking contrast between the records of these normal young and the mortality record, the frequency of easily recognized nervous symptoms of degeneracy, and the prevalence of gross deformities in the experimental races.

The external as well as internal factors are to be considered not only in individual or embryonic development, but also in heredity. And the present experiments now demonstrate for mammals that either the spermatozoon or the ovum may be experimentally injured or modified in such a manner as not only to give rise to (abnormal) subnormal development in the resulting embryo, but the effects of the injury may be transmitted from generation to generation, until an affected line actually fades out through degeneracy and sterility as a result of the transmitted condition.

Material and Methods

The animals used in the experiments have been ordinary vigorous guinea pigs of large size, particular care being taken to select animals less than one year old to begin with and good breeders.

At the beginning of the experiments alcohol was given along with the food, but the animals ate less and the food usually disagreed with them. It was then administered in diluted form by a stomach tube; this method was even more unsuccessful, disturbing digestion and seeming to upset the animals considerably. It is certain that alcohol given to animals through the stomach deranges their appetite and digestion to such an extent that the experimenter is unable to determine whether the resulting effects are due to the alcohol, as such, or to the generally deranged metabolism of the animal. When given in drinking water they take little or none of the water and the treatment is insufficient. For these reasons an inhalation method of treatment was resorted to early in the study, and, as far as experience goes, it has no serious disadvantages and does not complicate the conditions of the experiment.

This method may be merely described in brief for the convenience of the reader, since it has been fully recorded with illustrations of the fume tanks in previous publications. A fume tank of copper is made of sufficient size to supply breathing space for four or five guinea pigs at one time. The tank has four outlets, so that a definite amount of fumes may be passed through in a given time and the ventilation controlled. In this way each animal could be given a definite measured dose. The individuals, however, differ so much in their resistance to the treatment that it has been found better to treat all to about the same degree of intoxication. Such a physiological index is more reliable, since every animal may be affected to the same degree each day. For this purpose the animals are placed in the fume tank on a wire screen, and absorbent cotton soaked with alcohol is placed beneath the screen, so that they inhale the alcohol fumes arising from the cotton to saturate the atmosphere of the tank.

Ether was given in a similar manner. The animals are much more readily overcome by these fumes and must be carefully watched while inhaling even the most dilute doses.

To avoid handling the females during pregnancy, special treating cages are devised. An ordinary box-run with a covered nest in which the animal lives is connected by a drop-door with a metal-lined tank, having a similar screen arrangement, etc., to that of the general treatment tank. The pregnant animal may be driven daily into the tank and thus treated with alcohol fumes throughout her pregnancy without being handled in any way that might disturb the developing fetus.

Particular care is necessary in mating the animals in such an experiment, as the females are often slow to conceive and some of the F_2 and F_3 individuals of both sexes are not very prolific and in many cases are almost or quite sterile. Each female is kept in a separate run and the male is placed in with her just before the time of the expected heat period, ovulation, and he remains in her cage for from two to three weeks so as to be present at the second ovulation, provided the female was not made pregnant by the first mating. The ovarian cycle of the guinea pig as worked out by L. Loeb seems to correspond closely to what is found in mating experiences.

After mating, the male is removed from the cage and the female remains alone until the young are born. These are left with the mother for about fifteen days, then separated, and the female mated again. In this way the normal females may sometimes give as many as four litters per year, but the experimental animals breed much slower and it is difficult to get even three litters per year.

Direct Effects of the Alcohol Treatment on the Animals

Several of the guinea pigs have now been treated with the fumes of alcohol almost to the point of intoxication for six days per week for a period of five years. This is a considerable space in the life of a guinea pig, which probably would not often extend beyond six or seven years.

The animals are affected by the alcohol fumes in various ways; some of them are stupefied and become drowsy, while others become stimulated and excited and sometimes even vicious, constantly fighting and biting at the other animals in the fume tank. The fumes inhaled into the lungs pass directly into the circulation, so that the animals show signs of intoxication very soon after being put into the tank, yet the intake of alcohol is so gradual that they may remain for one hour or more without becoming totally anesthetized.

The mucosa of the respiratory tract is considerably irritated during the early stages of the treatment, but develops a resistance so that later little effect can be noticed. The cornea of the eye is greatly irritated, often becoming milky white and opaque during the first few months. In some cases this later clears and the animal is again able to see, though some of the animals treated for several years have remained entirely blind. The general condition of the animals under the fume treatment is very good. They all continue to grow if the treatment is begun before reaching their full size, and become fat and vigorous, taking plenty of food and behaving in a typically normal manner.

Some of the treated animals have died and others have been killed at different times during the progress of the experiment and their organs and tissues examined carefully and then studied microscopically. All have seemed practically normal. Tissues from several animals treated as long as three years have been examined and the heart, stomach, lungs, kidneys, and other organs present no noticeable conditions that might not be found in normal individuals. Alcoholized animals are usually fat, but there is no fatty accumulation in the parenchyma of any of the organs.

Several of the animals, both males and females, have been partially castrated during the experiments and the ovaries and testes have been found to be in a healthy condition, though certain possible changes may be present which are now being closely studied cytologically and experimentally.

The treated animals are, therefore, little changed or injured so far as their behavior and structure goes. Nevertheless, the effects of the treatment are most emphatically shown by the type of offspring to which the alcoholized individuals give rise, whether they be mated together or with normal individuals. The further significance of the nature of the effects is indicated by the quality of the subsequent generations descended from such an ancestry.

INFLUENCE OF THE TREATMENT ON THE DESCENDANTS OF ALCOHOLIZED ANIMALS

It may be well in the first place to consider the results of the experiments from a general standpoint and then to undertake an analysis of the reactions and conditions presented in the several generations and from the several lineal combinations. The records of the matings of the alcoholized animals in various pairs, the control or normal matings, and the matings of the F_1 and F_2 generations, the children and grandchildren of the alcoholized individuals are summarized in the general Table I. This table gives a record of all the matings of the kinds indicated up to July 1, 1915. A similar table was published two years ago, when the number of animals considered was much smaller and the actual indications from the results were less certain than now. On comparing this table with the former one, however, it will be seen that the continuation of the experiments has fully substantiated the results as previously recorded. The table now shows the records of 571 matings which produced 682 full-term young and 189 early abortions or negative results. These numbers are now of considerable magnitude in spite of the fact that the experiment is conducted on mammals which produce only small litters and breed slowly as compared with lower animal forms.

In the first horizontal line the record of pairing alcoholized male guinea pigs with normal females is given. This combination could only produce defective or subnormal young as a result of the injured male germ cells, since the ova are normal and develop in a normal untreated mother. This then is the definite test of the influence of the alcohol treatment on the germ cells.

Ninety such matings have in 37 cases given negative results; that is, failures to conceive, or early abortions. Thus 41 per cent. of the matings of such males were nonproductive, while less than 25 per cent. of normal matings under the same breeding conditions failed to produce full-term litters. Ten stillborn litters, each consisting of two young, twenty stillborn young, resulted from the 90 matings. While the 90 control matings gave only two stillborn litters, and in both cases these were unusually large litters of four individuals each, and they were probably dead on account of the fact that the mother could not give normal birth to so many offspring. The stillborn litters by the alcoholized fathers were all ordinary-size litters of two young. Thus, while 11 per cent. of the matings of alcoholized males resulted in stillborn litters, only 2 per cent. stillborn litters occurred from normal matings. Forty-three living litters were produced or a little less than 48 per cent. of the matings gave full-term living young, while 73 per cent. of the normal matings give living litters of young.

The 43 litters from alcoholic fathers contained in all 82 young, and 35, or almost 43 per cent., of these died soon after birth, while 66 similar litters from the control lost only 19 young, or 16 per cent., out of 118 individuals. Finally, then, from the 90 matings of alcoholic males with normal mates only 43 full-term litters resulted, consisting in all of 102 young; 55 of these, or 54 per cent., died at birth or soon after, and only 47 individuals, or 46 per cent., survived. Only about half as good record as the 78.5 per cent. surviving young from the matings of normal animals. Almost all of the offspring were very excitable, nervous animals and three of them showed gross deformities of the eyes, while no such conditions were found among any of the offspring of normal animals bred under identical conditions.

These records leave no doubt that the alcoholized male guinea pig is injured in such a way as to induce a decidedly bad effect upon the quality and mortality of his offspring when compared with the records from normal animals.

The second horizontal line of Table I shows the results obtained when alcoholized female guinea pigs are paired with normal males. In this case there is a double chance to injure the offspring. First through the influence of the treatment on the oocytes or the unfertilized ovarian egg, a direct effect on the germ cells comparable to the injury of the germ cells in the case of the treated males considered above. While in the second place, the developing embryo in the uterus of an alcoholized female may be directly affected by the strange substances contained in the blood and body fluids of the mother. Thus a defective individual may be produced as a result of development in an unfavorable environment or as a result of being derived from an injured or defective egg cell.

Thirty-three matings of alcoholized females with normal males have in seven cases, 21 per cent., given negative results or early abortions; this compares very favorably with the records of the control animals. Four stillborn litters consisting of three individuals each were produced. This is a record of 12 per cent. stillborn litters against only 2 per cent. from normal matings. The alcoholized females gave birth to 22 living litters containing 44 young, and 23, or 52 per cent., of these died, only 48 per cent. surviving against 84 per cent. survivals among the young of similar control litters. The records of the matings of alcoholized females compare very unfavorably with the record of the control matings. Yet the behavior of the alcoholized females is very little, if any, worse than the records shown by the alcoholized males in spite of the double chance the female has to injure her young.

The third horizontal line of the table indicates the results obtained when alcoholized males are paired with alcoholized females. Here there is every chance for the treatment to show its effect. The percentage of early abortions or negative results is very high, about 50 per cent. more than double that of the control matings. Ten per cent. of the matings produced stillborn litters each consisting of two young. Only 17 living litters were born out of 41 matings, about 41 per cent., against 73 per cent. living litters from 90 control matings. The 17 living litters contained only 26 young, and 12 of these, or 46 per cent., died soon after birth, while but 16 per cent., or one third as many, of the control offspring died out of a total of 118 individuals. The data from the double alcoholic matings is, therefore, extremely bad in the light of normal matings from the same animal stocks bred under exactly the same cage and food conditions.

The fourth horizontal line summarizes the records of all the matings of directly alcoholized animals. In all 164 such matings have been made; 64 of these, or almost 40 per cent., gave negative results or early abortions. Eighteen stillborn litters occurred, consisting of 40 individuals against only two questionable stillborn litters from 90 control matings. Eighty-two, or only 61 per cent., living litters were born, consisting of 152 individuals, 82, or 54 per cent., of which survived and 70, or 46 per cent., died soon after birth; in all 110 full-term young died, while only 82, or 42 per cent., of the total 192 full-term young resulting from the 164 alcoholic matings survived. On the other hand, out of a total of 126 full-term young from only 90 control matings, 99, or 78.5 per cent., survived. The control matings were far more prolific than those of the alcoholized animals and the condition of the young as indicated by the mortality record was far superior to that of the alcoholic offspring.

The fifth line records the outcome of 90 control matings which have been scattered through the entire progress of the experiment under exactly the same conditions and from the same animal stocks as the experimental matings. Eighty-four per cent. of the young in the 66 living litters resulting from the matings of normal animals have survived and all are strong, healthy individuals; in not one instance do they show an indication of nervous degeneracy or any type of recognizable structural deformity, while such degeneracy as well as deformities are extremely prevalent among the offspring and descendants of the alcoholized animals. One other point to be mentioned in considering the records of the

TABLE I

EFFECTS OF ALCOHOL ON THE DESCENDANTS OF TREATED ANIMALS

Condition of the Animals	Number of Matings	Negative Result of Early Abortion	Stillborn Litters	Number of Stillborn Young	Living Litters	Young Dying Soon After Birth	Total Dead	Surviving Young
Alcoholic ♂ × norm. ♀	90	37	10	20	43	35, 1 c.e.	55	47, 2 o.e.
Norm. ♂ × alcoholic ♀	33	7	1	12	22	23	35	21
Alcoholic ♂ × alcoholic ♀	41	20	1	8	17	12	20	14
Summary	164	64	18	40	82	70	110	82
Control norm. ♂ × norm. ♀	90	22	2	8	66	19	27	99
♀ treated during pregnancy .	1	0	0	0	1	1	1	7
Second generation × norm. .	46	10	3	8, 6 c.e.	33	29, 2 par.	37	25, 3 c.e.
Second gener. × alcoholic	53	16	8	17, 1 d.e.	29	22, 3 d.e.	39	28
Second gener. × second gener. ...	95	29	7	16	59	43, 2 par., 6 d.e.	59	52, 3 d.e., 1 one e., 1 eyeless
Third gener. × third gener.	48	20	7	14, 1 d. legs	21	19, 1 par., 6 d.e., 2 eyeless	33	13
Third gener. × second gener.	33	15	1	8	11	16, 1 par., 1 c.e.	24	7
Third gener. × normal	17	3	1	8	10	5	13	7
Third gener. × alcoholic	3	1	0	0	2	2	2	1
Second, third gener. × second, third gener.	18	9	2	6	7	6	12	1

control matings is the fact that from 90 matings only two stillborn litters were produced and, as mentioned above, both of these litters were of so large a size that the mothers seemed unable to successfully deliver them and one of the mothers failed to recover from the process and died a few days later. These two cases make the control records appear worse than they actually should, but in spite of this the control matings have given data equally as good as those generally obtained by careful breeding experiments with vigorous normal stocks. The stock in these experiments is unquestionably good, as the control matings very readily show.

Four normal females were mated and then treated with alcohol throughout their periods of pregnancy and, as the sixth horizontal line of the table indicates, such a treatment was not at all injurious in these particular cases. It actually happened that some of these young were unusually vigorous. The numbers are very small, but this is a direct test, and if such a treatment were really decidedly effective in its action on the embryo or fetus *in utero* these eight young animals should have at least shown some response. It is very possible that after the treatment has been continued for a long time, a year or more, that the mother then presents a uterine environment unfavorable for normal development, since the offspring of such individuals are almost always subnormal. In these cases, however, the inferior quality of the offspring may be due to the action of the alcoholic treatment on the ovarian germ cells rather than the direct environmental effect on the developing embryo or fetus, there is no way at such a stage to separate the two possible effects.

The next three horizontal lines, seventh, eighth and ninth, give the data resulting from the matings in various combinations of the F_1 animals, that is, offspring from alcoholic parentage, but which are not themselves treated with alcohol. The records of these non-treated F_1 individuals are most instructive for an understanding of the actual influences of the alcoholic treatments.

When such F_1 animals are paired with normal individuals the seventh line shows that almost 22 per cent. of the matings failed, which is not a bad record. The proportion of stillborn litters, however, from the F_1 by normal combination was three times as great as from normal matings and 75 per cent. of the stillborn young produced showed gross defects of the eyes, having opaque lenses or typical cataract conditions, while not one of 126 young from normal matings has shown this or any other noticeably abnormal structure. Thirty-three living litters were produced containing in all 54 individuals, 29, or 54 per cent., of which died soon after birth, while 25 survived. Two of those dying soon after birth were paralyzed and unable to walk, while three of the 25 survivors have defective opaque eyes, cases similar to that illustrated by Fig. 1, and many show different nervous symptoms. Thus of 62 full-term young produced by F_1 animals with normal mates, only 25, or 40 per cent., survived for more than a short time after birth, and 12 per cent. of these have gross defects and more than half of them are nervous, excitable individuals, which when mated with normal animals or in any other combination always give very poor quality offspring, if any at all.

The eighth line shows the records of 53 matings between F_1 animals and alcoholics. This combination again gives data comparing most unfavorably with the control and in some ways even worse than the records of matings between two alcoholic animals. Fifteen per cent. of such matings produced stillborn litters! Only one combination gives a worse record of stillborn that is, matings among F_2 animals. Almost half of the young in the living litters died and here again some were deformed. Deformities are strikingly more abundant among the offspring from F_1 and F_2 parents than from the directly alcoholized animals.

The record of 95 *inter se* matings of F_1 animals is shown in the ninth line. Thirty per cent. of such matings gave negative results or early abortions, over 7 per

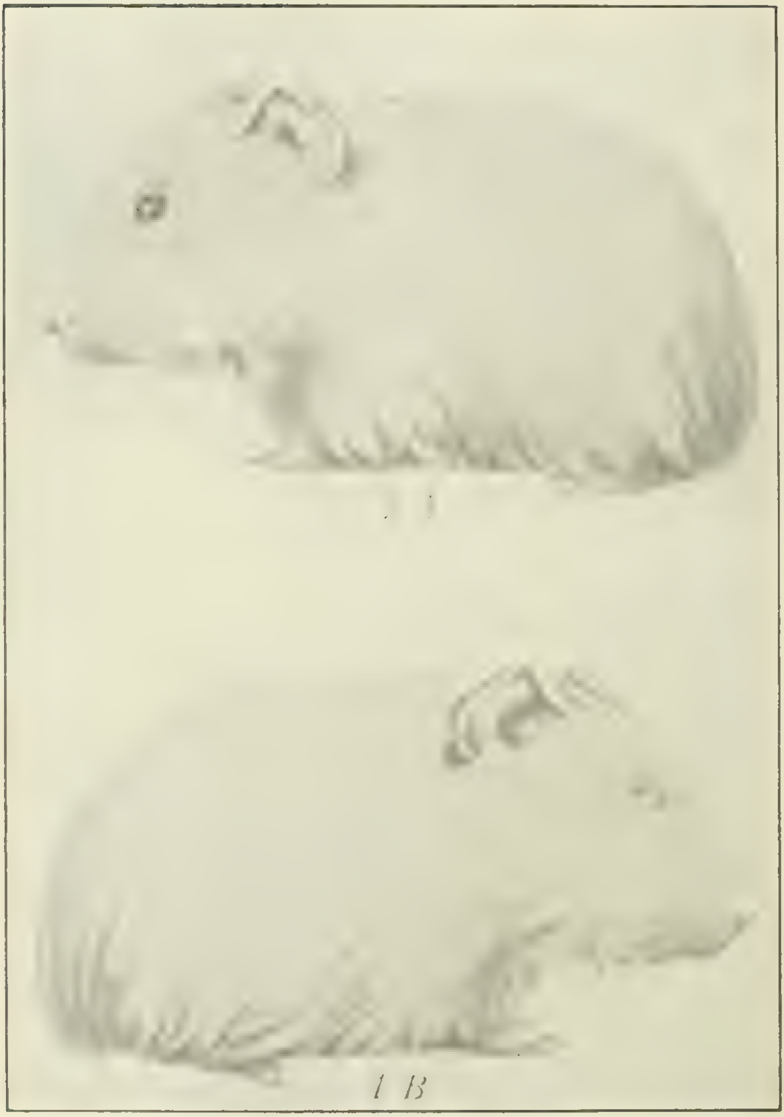

FIG. 1, *A* and *B*. 271 F_2 ♀ (one in litter) (AA)(AN). Both paternal grandparents and the maternal grandfather were alcoholic, no inbreeding. The right eye is smaller than the left and has been entirely opaque since birth. This animal, almost two years old and vigorous, is entirely sterile.

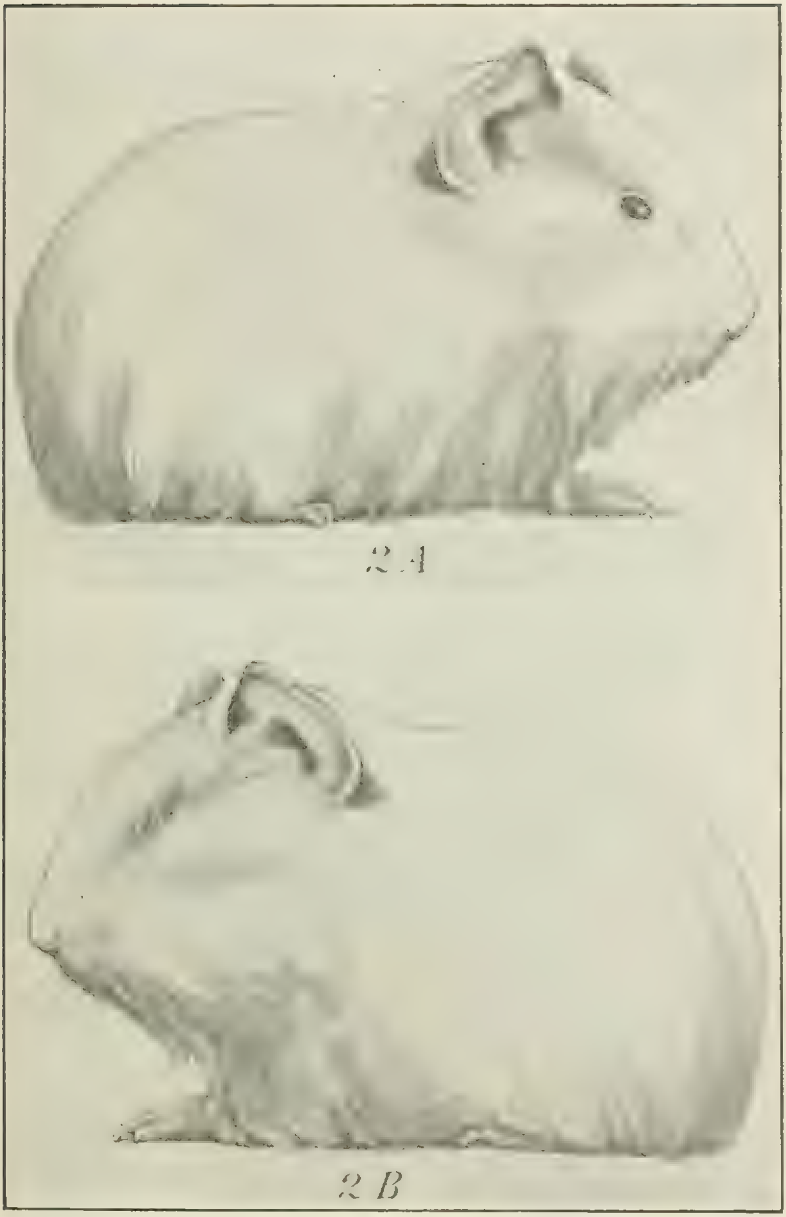

FIG. 2, *A* and *B*. 307 F_2 ♀ (one in litter). Inbred, from brother and sister offspring of an alcoholic male (AN) (AN). The eye of one side normal, the other eye ball apparently absent on living examination. A typical monster monophthalmicum asymmetricum. This animal now 21 months old is completely sterile.

cent. stillborn litters and 62 per cent. living litters. Little less than half of the living young died soon after birth, in all 43, nine of which, or more than one in five, 21 per cent., were paralyzed or deformed; the figures in Plates I and II illustrate the paralytic conditions. Fifty-two of the offspring survived, three with deformed eyes, one with one eyeball completely absent, monster monophthalmicum asymmetricum (Fig. 2, 307 ♀), and almost all of the 52 are very nervous, excitable animals which when bred give rise to deformed or highly degenerate offspring.

The offspring from the F_1 animals mated in any combination are generally far below the normal in power to survive and in quality of structure. When compared with the offspring from directly alcoholized parents, the offspring from the F_1 combinations show an equally bad mortality record and a very much higher proportion of paralyzed and deformed individuals. The 95 matings *inter se* of F_1 animals demonstrate conclusively that such individuals carry defective or abnormal germ cells which give rise to defective developmental products. These degenerate F_2 offspring owe their subnormal condition to the effects of the action of the alcohol treatment upon the germ cells of their grandparents which have been transmitted to them through their parents. In other words, the carriers of hereditary qualities have been modified in the first parental generation, and the effects of this modification are expressed in their offspring F_1, and also in their grandchildren, the F_2 generation.

The next line of the table, the tenth, indicates further how the effects of the original modification are transmitted to the great grandchildren or through three generations since the injury. Forty-eight *inter se* matings of F_2 animals gave the results here shown. Almost 42 per cent. of the matings gave negative results or early abortions, the poorest record in this respect shown in the entire table. About 15 per cent. of such matings gave stillborn litters, 7 in 48 matings, which is remarkably high when compared with any of the above combinations.

The hind legs of one of the stillborn young were deformed in the peculiar manner illustrated in Figs. 4 and 5.

Twenty-one living litters were produced, containing in all 32 young; 19 of these, almost 60 per cent., died soon after birth, and only 13 survived. One of the 19 that died was paralyzed and unable to stand, while 8 of them, a strikingly high proportion, were grossly deformed. Six

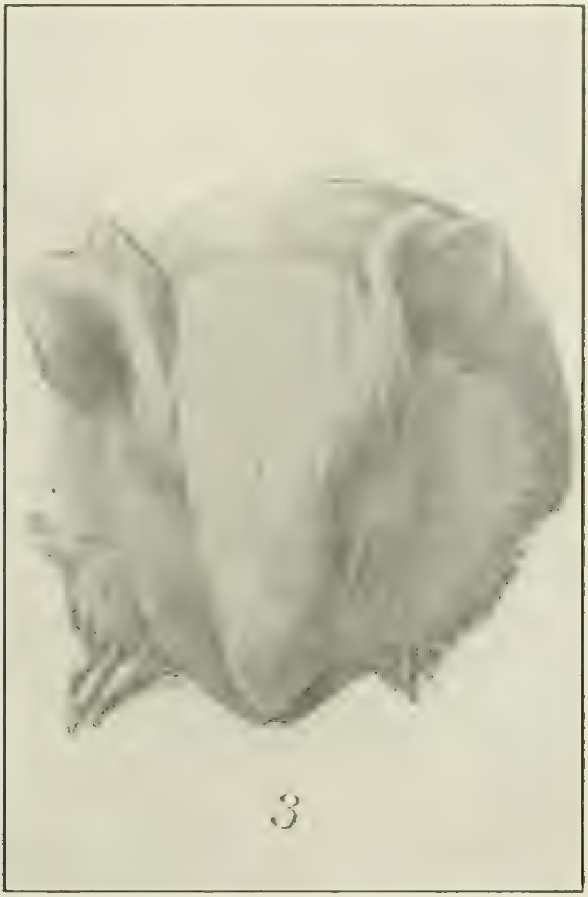

FIG. 3. F_3. Two in litter, both same condition, three normal great-grandmothers and three alcoholic great-grandfathers. The parents were single first cousins. Both animals completely eyeless, also with paralysis agitans, one died the second and the other the third day after birth, typical anophthalmia. One brain no indication of optic nerve, the other slight processes.

had one or both eyes deformed (Figs. 1 and 2), and two were anophthalmic monsters, being completely without eyeballs, optic nerves, optic chiasma or any gross signs of optic tracts (Fig. 3). The brains are now being studied in sections. Figs. 1 to 3 illustrate animals showing the different eye conditions—asymmetrical eyes, monstrum monophthalmicum, and anophthalmic monsters. Figs. 5 and 6 of Plate III illustrate the brains of a normal and an anophthalmic specimen for a comparison of the condition of the optic nerves, etc.

Forty-six full-term young were produced by the F_2 matings, but only 13 of these, or just 28 per cent., were able to survive, while about three times this proportion, or 78.5 per cent. of the full-term young from control matings, survived as vigorous healthy individuals. The 13 living F_3 animals are all rather weak and degenerate and almost completely sterile according to a considerable number of careful matings with strong, fertile guinea pigs. The alcoholic race seems at this stage of the experiment about to fade out in the fourth generation, while normal control lines from the same original stocks have passed far beyond this generation, continuing to breed normally and showing no signs of degeneracy, and never in any case giving rise to a grossly deformed animal.

The eleventh line indicates again the very decided effects transmitted by the descendants of animals which had suffered a modification of their germ plasm by the alcoholization of their tissues. In 33 cases F_1 and F_2 animals were paired together. Fifteen of these matings gave negative results or early abortions, while about 12 per cent. of the matings resulted in stillborn litters of two young each. Only 14 living litters were produced by the 33 matings; these contained in all 23 young, only 7 of which survived. Thus from a total of 31 full-term young only 7, or about 22 per cent., were capable of surviving. All of these young animals are nervous and weak and several offspring from these combinations were deformed.

When F_2 animals are mated with normal individuals the results are very little if any improved over the two above combinations. Seventeen such matings gave only three failures or early abortions, but a high proportion, 23 per cent., of stillborn litters arose, while 10 living litters, consisting of only 12 individuals, were born. In all 20 full-term young were born and only about one in three of them survived. In this experiment, although one mate was a normal animal, the F_2 mate carried germ cells of so inferior a quality that the output of the combination, admitting the numbers are small, leaves no doubt of the transmission, *through three generations*, of defective conditions induced by alcoholizing the great grandparents of the offspring on only one side of the family, or in only one of the parental lines.

The last line of the table gives the records of mixed combinations of F_1 and F_2 individuals, and here the data are closely similar to those obtained from other combinations of these animals; only about 25 per cent. of the full-term young born are capable of surviving, while 78.5 per cent. of the control young are living.

Briefly, then, 571 matings tabulated in Table I, the records to July 1, 1915, have given rise to 682 full-term young, as well as a large number of premature abortions. A careful study of all these young animals extending over a period of five years has afforded data which convincingly show that the treatment of either the male or the female guinea pig with fumes of alcohol affects the quality of the offspring to which these animals give rise even when paired with normal mates. And further, the changed quality of the offspring is subsequently transmitted through succeeding generations with even more severe marks of degeneration and deformity than those exhibited by the offspring of the directly treated animals.

Other combinations and back crosses are now in progress which are fully in line with the above, but which have not yet afforded sufficient analytical data to record.

The defects caused by the alcohol treatment seem to be largely confined to the central nervous system and organs of special sense. Paralysis agitans is very common among the F_1, F_2 and F_3 animals. Paralyzed limbs are often observed, the animals being unable to stand or walk (Plates I and II). The eye is also a peculiarly sensitive indicator and presents in the various descendants of alcoholized individuals all degrees of degeneration—opaque cornea, cataract or opaque lenses, small defective eyes, complete absence of one eye and finally complete absence of both eyeballs—anophthalmic monsters. In the latter case the extrinsic eye muscles, the third, fourth and sixth nerves, the lachrymal glands and other struc-

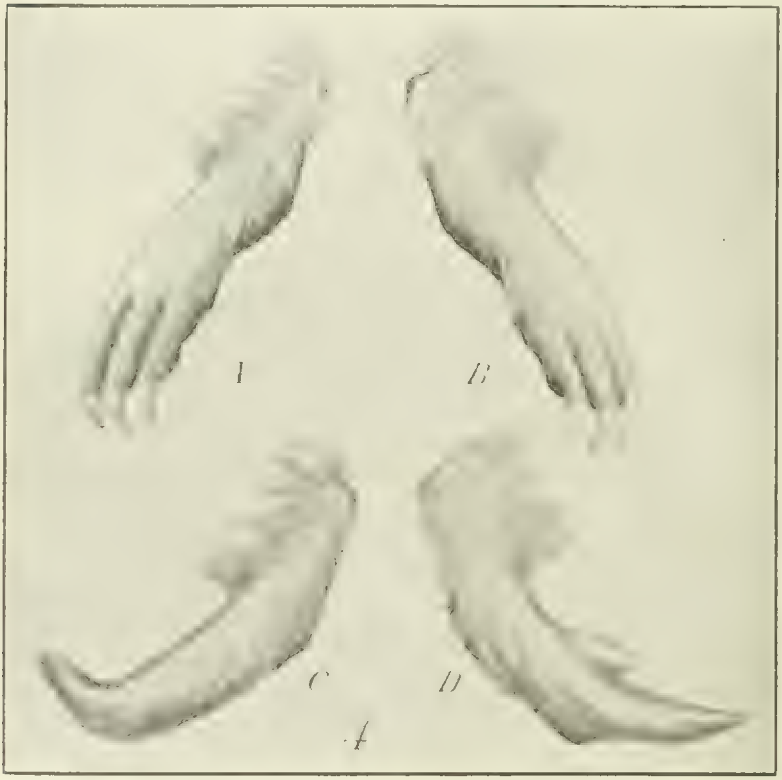

FIG. 4. Hind feet of No. 488 $F_{2,3}$ ♀. All great-grandparents were alcoholic as well as the maternal grandparents. Inbred from mother by son. This animal was one of a litter of two stillborn. The left hind foot, *C*, had only one toe and the right, *D*, one toe and a stump, *A* and *B*, normal right and left hind feet.

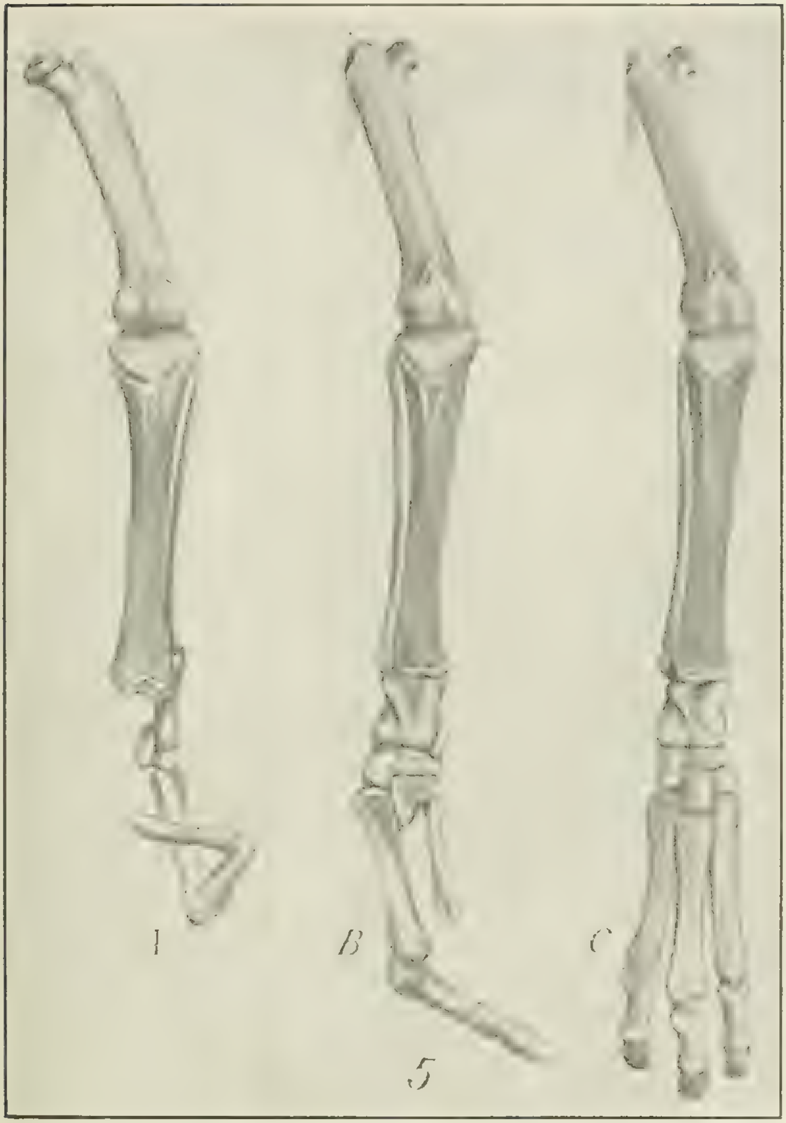

FIG. 5. The skeleton of the two limbs from Fig. 4 shows the left foot, *A*, to have only one metatarsal and toe, the third, and three tarsal bones. The right foot, *B*, has the third toe also and the first metatarsal with the tarsus almost complete. *C* shows the normal skeleton of a right hind limb with the three toes and seven tarsal bones.

tures of the orbit are present, though the eyeball is completely wanting.

Not only are the above congenital eye defects present, but in several instances members of the alcoholic lines have become blind during the first year or year and a half after birth, whereas in our control this has never occurred.

The several illustrations referred to above show specimens exhibiting these various defects. Figs. 1, 2 and 4 of Plates I and II are photographs of animals of indicated lineage which show paralytic conditions. Figs. 4 and 5 illustrate defective extremities. Figs. 1 to 3 show various degrees of defective eyes and absence of eyeball.

It is peculiarly interesting to find these particular eye conditions exhibited by the descendants of alcoholized animals, since, as Stockard ('10) has previously shown, closely similar eye conditions are obtained in great numbers by directly treating the eggs of fish with solutions of alcohol; and like conditions were also obtained, though not so consistently, by treating hens' eggs ('14) with alcohol fumes either before or during incubation.

The table just considered gives only a general idea of the experiment and is in no way analytical. We shall now attempt to analyze these data in such a manner as to determine the influence of internal factors, as, for example, inbreeding on the results. The influence of the size of the litter on the quality of the offspring. The behavior of F_1 and F_2 individuals derived from different lines, and whether there is a difference in the effects on male and female animals, and the manner of transmission of these effects.

(To be continued.)

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A FURTHER ANALYSIS OF THE HEREDITARY TRANSMISSION OF DEGENERACY AND DEFORMITIES BY THE DESCENDANTS OF ALCOHOLIZED MAMMALS. II

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THE INFLUENCE OF INTERNAL AND EXTERNAL FACTORS ON THE QUALITY OF THE OFFSPRING

Table II gives the relationship between the size of the litters and the mortality of the descendants from different combinations. It brings out in a way the variable internal and external factors to be considered in interpreting the conditions of the members of the numerous litters of animals. The external factor considered in the table is one of nutrition or environment, depending upon the number of young developed in the uterus at any one time. The table indicates the influence of an internal factor, the germ plasms concerned in mating related or non-related animals. Four combinations are considered: pairs of normal non-relatives, pairs of alcoholic non-relatives, pairs of normal relatives, and pairs of alcoholic relatives.

The first vertical column shows that in mating together normal non-related guinea pigs of the stocks used in these experiments the average litter contains 1.96 individuals. Fifty-one and eleven hundredths per cent. of the young were found in litters of two, and 20 per cent. of the animals occurred in litters of three. Fifteen and fifty-five hundredths per cent. of the animals were born in litters of only one young, and 13.33 per cent. in litters of four individuals.

The next space below in the table shows the number and percentage of individuals living over three months

TABLE II

The Size of Litters and Mortality of Descendants from Different Combinations

	Normal Lines 90				Alcoholic Lines 401				Normal Inbred 30				Alcoholic Inbred 135			
	1 in litt.	2 in litt.	3 in litt.	4 in litt.	1 in litt.	2 in litt.	3 in litt.	4 in litt.	1 in litt.	2 in litt.	3 in litt.	4 in litt.	1 in litt.	2 in litt.	3 in litt.	4 in litt.
Total number.	14	46	18	12	84	214	87	16	12	18	0	0	38	76	21	0
	(15.55%)	(51.11%)	(20%)	(13.33%)	(20.94%)	(53.36%)	(21.69%)	(3.99%)	(40%)	(60%)			(28.14%)	(56.29%)	(15.55%)	
	(Average number of young per litter 1.96)				(Average number of young per litter 1.79)				(Average number of young per litter 1.43)				(Average number of young per litter 1.62)			
Lived over 3 months....	12	40	11	4	54	109	19	3	12	12	0	0	29	20	0	0
	(85.71%)	(86.95%)	(61.11%)	(33.33%)	(64.28%)	(50.93%)	(21.83%)	(18.75%)	(100%)	(66.66%)			(76.31%)	(26.31%)		
	(All together 74.44%)				(All together 46.13%)				(All together 80%)				(All together 36.29%)			
Died within 3 months....	2	6	7	8	30	105	68	13	0	6	0	0	9	56	21	0
	(14.28%)	(13.04%)	(38.88%)	(66.66%)	(35.71%)	(49.06%)	(78.16%)	(81.24%		(33.33%)			(23.68%)	(73.68%)	(100%)	
	(All together 25.56%)				(All together 53.86%)				(All together 20%)				(All together 63.70%)			
Deformed...	0	0	0	0	2	9	10	0	0	0	0	0	2	14	4	0
					(2.25%)	(4.20%)	(11.49%)						(5.26%)	(18.42%)	(19.04%)	
					(All together 5.23%)								(All together 14.81%)			
Undersize (less than $\frac{2}{3}$ of the average weight)....	0	0	0	0	1	4	3	2	0	1	0	0	1	4	0	0
					(1.19%)	(1.86%)	(3.44%)	(12.50%)		(5.55%)			(2.89%)	(5.26%)		
					(All together 2.49%)				(All together 3.33%)				(All together 3.70%)			

in the different-size litters. Almost 86 per cent. of the individuals born one in a litter lived, and about 87 per cent., 40 out of 46, of those born two in a litter lived. When there were three in a litter, however, only 61 per cent. lived and of those born four in a litter, it happened that only one third of them survived, though there were only a few in all. Of the total number of young from normal non-related parents 74.44 per cent. lived. Judging from these statistics litters of one or two young are the most vigorous and individuals born in litters of three or four are not so likely to be strong and long-lived.

The next space below gives the mortality records, which, of course, is merely another way of bringing out the above statements. The space following contains the number of deformed animals, but from the normal matings not one such individual has been produced. The last space gives the number of small-size or dwarf specimens also, none of which occur among these litters from normal non-related parents.

The second vertical column contains a similar analysis of the influence of the size of the litter on the mortality and condition of the young born from non-related alcoholic parentage. This not only includes the offspring from directly treated animals, but also other matings of non-relatives belonging to the alcoholic lines. Here again the majority of all the young, 53.36 per cent., are born in litters of two. Litters containing three are next in frequency, followed by litters of only a single individual. Of the total number of offspring produced by alcoholic parents 21.69 per cent. occurred in litters of three, and only about 4 per cent. of the offspring were members of litters of four individuals. The average number of young in the litters from these animals is 1.79, somewhat smaller than from normal matings.

The space below shows that in all only 46.13 per cent. of these young survived, whereas more than half as many more, or 74.44 per cent., from normal parentage lived over three months. The most vigorous animals are those born only one in a litter. Sixty-four and twenty-eight hundredths per cent. of them lived. While about 51 per cent. of the two-in-a-litter individuals survived, only about 22 per cent. of the young born three in a litter were capable of surviving, and only 18.75 per cent. of the individuals from the litters of four lived more than three months. These figures indicate that the offspring from similarly injured parents are more capable of survival when born in a small litter of one or two than when contained in larger litters of three or four.

This is not on account of the fact that the treated or degenerate mother is more incapable of nourishing the larger litters, since the same is true of the larger litters from normal mothers, as shown by the previous column. The fact is that all young of large litters tend to be small and weak at birth, whereas a single young is far better accommodated. For these reasons it is always of importance to know the size of the litter in which an animal was born in estimating the degenerate qualities it may possess as compared with the qualities of another individual. For example, one animal may appear larger and stronger than another, and yet when bred will give rise to more degenerate offspring than the weaker individual. Although having a vigorous body, its germ-cell complex was not so good as that of the weaker animal, from a larger litter which produces better offspring. Therefore, the small weak males bred to normal females do not always give the poorer results when compared with the matings of stronger males and normal females.

The next space is the reverse of the one above and shows the percentages of mortality among the offspring derived from alcoholic non-relatives. More than half of the young, 53.86 per cent., from these combinations die soon after birth, a mortality record just twice as high as that of the control animals.

The next space shows the frequency of deformities among such young. Here it is again clearly indicated that the animals born one in a litter are better than those

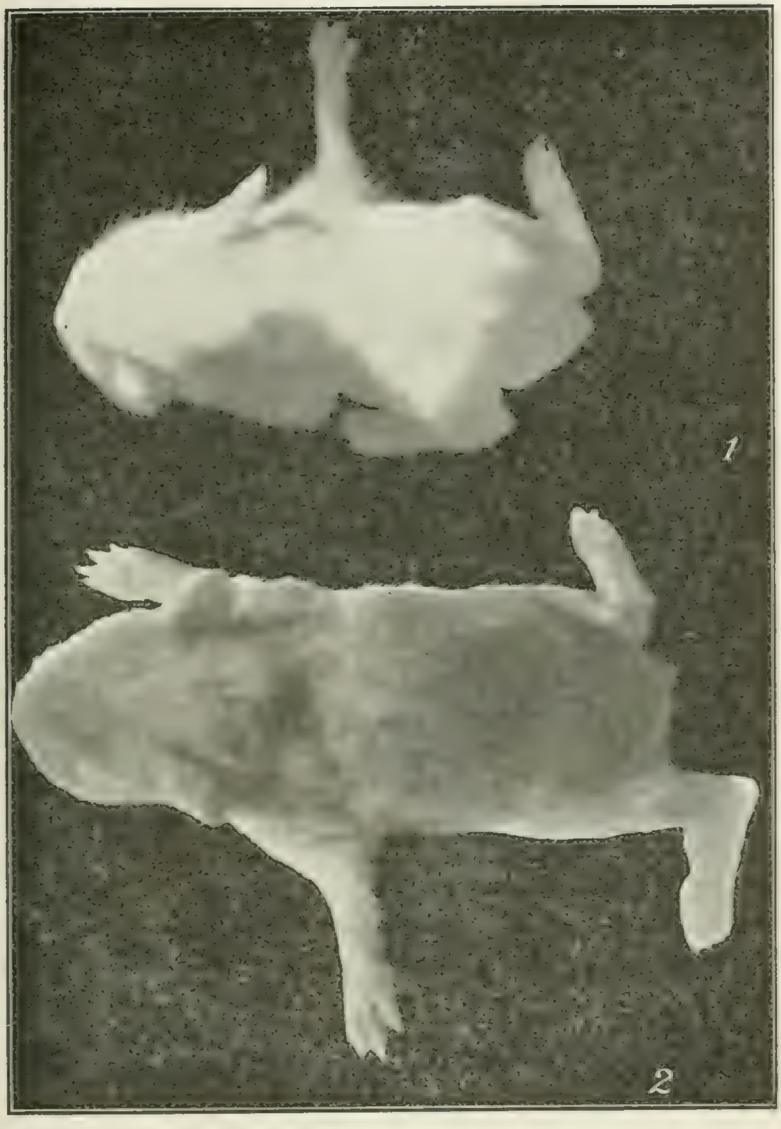


FIG. 1. 521 albino F_2 ♀ (two alcoholic grandmothers, both grandfathers normal). Lived only one day after birth; the meninges of the brain were filled with blood. Gross tremor and complete paralysis of right side. Cataracts, both crystalline lenses being entirely opaque. The photograph shows the outstretched paralyzed right extremities while the left legs are held in a normal position in their effort to support the body. (Birth weight, 54 grams.)

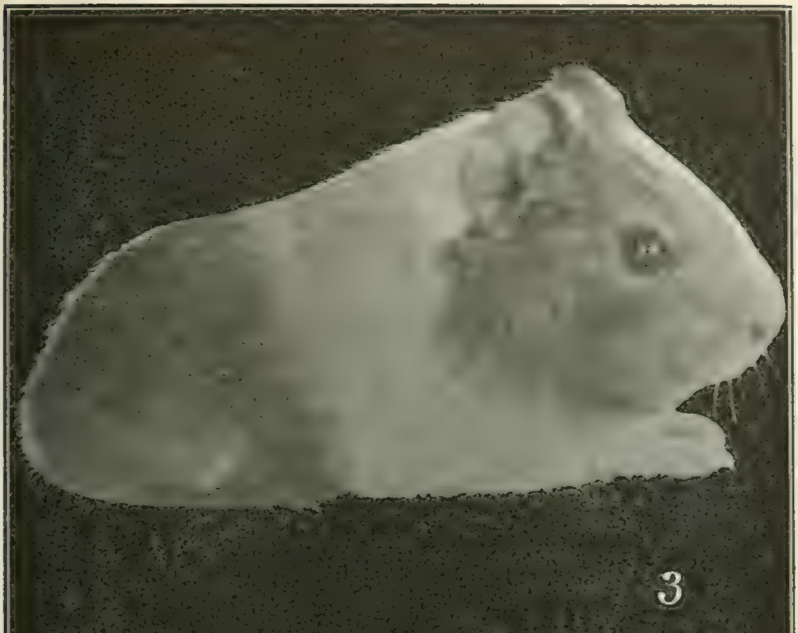
FIG. 2. 506 mouse and yellow $F_{2,3}$ ♂ (two paternal great-grandmothers and the maternal grandmother alcoholic, slightly inbred). Gross tremor and complete paralysis of left side so unable to walk. Cornea of right eye opaque. Photograph shows the powerless condition of the outstretched left legs with the right legs attempting to support the body. (Birth weight, 57 grams.) The two figures are at different magnifications.

from litters of two, which are in turn better than the members of the litters of three individuals. Only 2.25 per cent. of the 84 individuals born in litters of one were deformed. While 9 of the 214 individuals born two in a litter, or 4.2 per cent., almost twice as many, were deformed. And 10 of the 87 animals born three in a litter were deformed, or about 11.5 per cent., which is almost five times higher than the number of deformities found among the animals born in litters of single individuals. Among the descendants of alcoholic non-relatives there was in all 5.23 per cent. of deformed specimens, whereas not one deformed animal arose from similar normal matings.

The last space of this column indicates the number of dwarf or undersize animals produced in the different litters from non-related alcoholic lines. Among the 84 animals born one in a litter only a single individual was of unusually small size. The 214 animals born in litters of two were all of average size except four, or stated exactly, 1.86 per cent. of them were undersize. In the litters of three 3.44 per cent. of the animals were small, while 12.5 per cent. of the members of litters of four were small specimens. Here again it is shown that the members of large litters are not so uniformly up to the standard of size and vigor as animals born in litters of only one or two individuals.

The third vertical column gives a similar analysis of the few normal inbred individuals which have been produced during the time of the experiment. There are not many such matings, as a general effort has been made to avoid inbreeding the control animals since this might be considered to vitiate the results.

The few young produced by inbred normal matings have all been in litters of only one or two offspring, so that the size of the litters averages only 1.43 individuals. The size of the litters is, therefore, smaller than from either the non-related normal or alcoholic animals. Eighty per cent. of the young have survived, more, however, from the



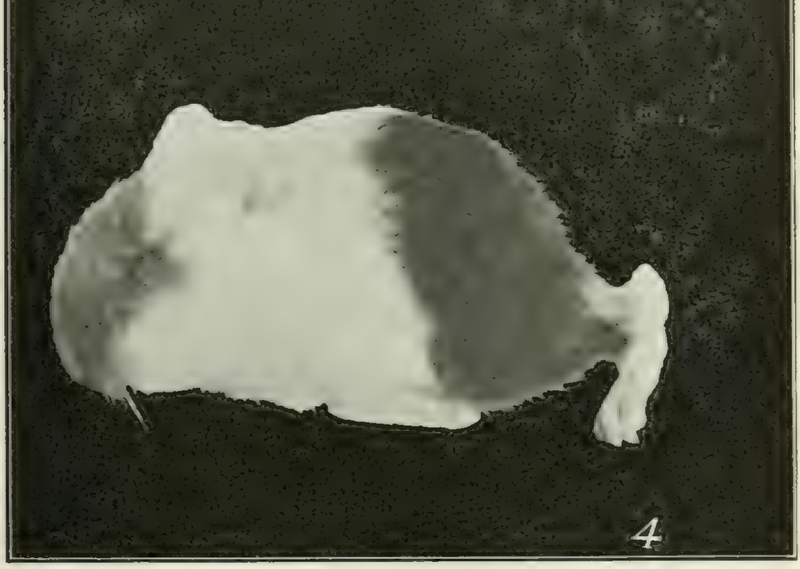


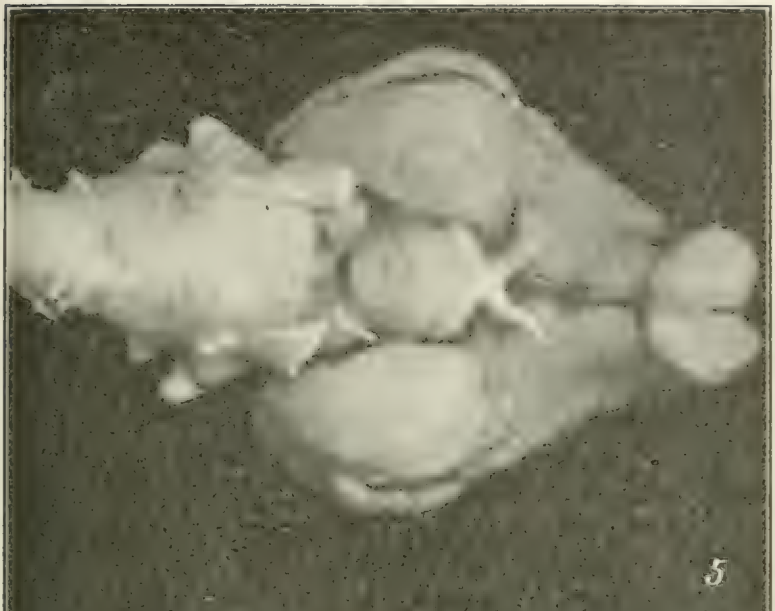
FIG. 3. 701 agouti, yellow and white, normal F_3 ♂. A normal animal from the fourth generation of the control; slightly inbred, natural size. Birth weight, 63 grams.)

FIG. 4. 599 black, white and red. F_3 ♂. A degenerate animal from the fourth generation alcoholic lines; no inbreeding; the paternal grandmother had both parents alcoholic and the maternal grandfather had both parents alcoholic. So there were two alcoholic great-grandmothers and two alcoholic great-grandfathers; the other four great-grandparents were normal and one grandmother and one grandfather had no alcoholism in their ancestry. The parents were ♂ (NN) (AA) ♀ (AA) (NN). The animal lived seven days and died in convulsions. The photograph shows the front limbs bent under the body and the animal is unable to raise the head. It weighed only 35 grams at the time of death, having lost 7 grams. While the above normal animal weighed 63 grams, actually a little small, at birth and all normal animals increase in weight rapidly from that time.

litters of one than from the litters of two. Not one deformed animal has resulted from these normal inbred matings and only one individual of the thirty was less than two thirds the average weight. The few normal inbred young here considered are then equally as good as the young from normal non-relatives and necessarily superior to the alcoholic lines. Judging from the results of others, there is little doubt that a more extensive inbreeding might produce deleterious effects.

The last vertical column indicates the effects of inbreeding alcoholic animals and their descendants. This combination shows the poorest quality offspring found in the table. Here again the members of the larger litters are at a disadvantage when compared with those born in the small litters. The average number of young in a litter is 1.62, somewhat smaller than the litters produced by mating alcoholic non-relatives. Thirty-eight litters contained a single individual each, the same number of litters contained two individuals, while only seven litters consisted of three young, and these were the largest litters produced. The inbred alcoholic animals, therefore, have a tendency to produce a large proportion of small litters and this tendency aids in strengthening their offspring.

Of the 135 young resulting from these matings, only 36.29 per cent. of them survived; this is the poorest life record shown by any combination. Of those born only one in a litter, however, 76.31 per cent. survived, which is a record equal to the average of the control. Therefore, even in this very bad combination, alcoholic inbreeding, when only one young is produced at a litter by an animal ordinarily capable of producing two or more, this one young is so well nourished and accommodated that it is somatically vigorous. Yet on breeding such individuals it almost always happens that very inferior offspring result. The germ cells, at any rate, may possibly be stronger than those in the weaker individuals which occurred in litters of two or three. Only 26.31 per cent. of the animals born in litters of two were capable of sur-



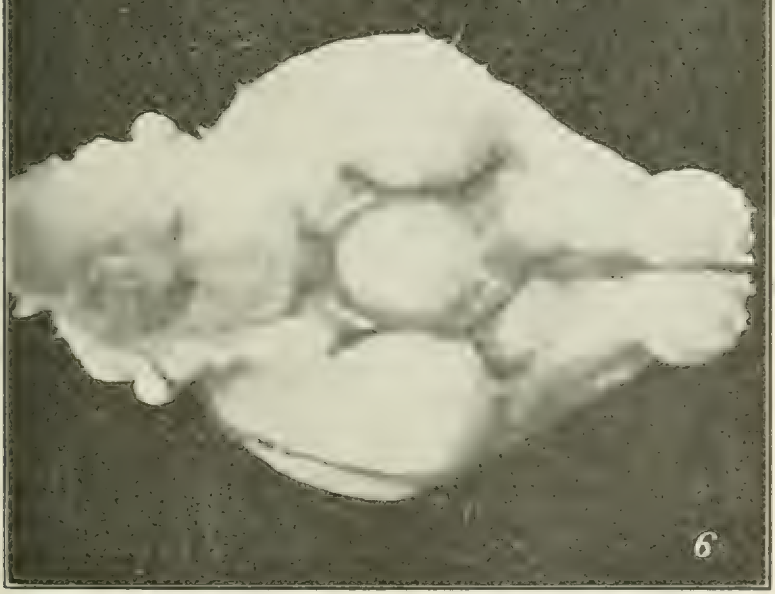


Fig. 5. Ventral view of a normal guinea-pig brain killed three days after birth. The optic nerves, optic chiasma and optic tracts passing along the tuber cinereum are distinctly seen.

Fig. 6. Ventral view of the brain of an anophthalmic monster from alcoholic great-grandfathers. There are no optic nerves, chiasma or tracts; the only indication of optic parts being two small membranous processes about in the position of the optic chiasma.

viving. The mortality here happens to be about three times higher than among the single-litter individuals. And further, not one of the 21 specimens born in litters of three lived. Among the offspring from the alcoholic inbred lines, judging from the numbers now available, the difference between the vitality of individuals born in litters of one and those born in larger-size litters is most striking.

The space below is the reverse of the one just considered and gives the percentage of young dying in the different litters. Only 23.68 per cent. died from litters of one individual, while 73.68 per cent. died in the litters of two individuals, and every one, 100 per cent., of the animals born in litters of three died within three months and usually within a few days.

The proportion of deformed animals occurring in the different-size litters again emphasizes the same differences in quality. All together 14.81 per cent. of the 135 individuals were grossly deformed; this is the highest percentage of deformed animals occurring in the several combinations represented in the table.

Of those animals born one in a litter only 5.26 per cent. were deformed; of those born two in a litter 18.42 per cent., or more than three times as many, were deformed, and of those specimens born in litters of three 19.04 per cent., or about one in five of them, were grossly deformed.

The proportion of deformities, therefore, conforms to the mortality records, being very much higher in the larger litters, and not unusually high among the individuals born one in a litter as compared with the average percentage of deformities from alcoholic non-relatives. Therefore, the bodily quality of the offspring is not materially worse from alcoholic inbred animals than from matings of alcoholic non-relatives, provided only one individual is born in the litter. But when more than one individual occurs in the litter, the alcoholic inbred combination is the most disastrous for the vitality and form of the offspring of all the combinations considered.

The last space shows that 3.7 per cent. of these offspring were less than two thirds the normal size. This again compares unfavorably with the other combinations, and here also the individuals born one in a litter show a superiority over those born in litters of two.

From a consideration of this table it may be concluded that the vigor of a guinea pig varies inversely with the size of the litter in which the animal is produced, and this is equally true whether the animal is born from normal or alcoholic parentage. However, the differences between the mortality of animals born in litters of one, two or three from normal parentage are not nearly so great as comparable differences between the members of the small and large litters from alcoholic lineage. For example, the difference in mortality between normal animals in litters of one or two is about 1 per cent., or scarcely any; between these and the mortality of specimens born three in a litter there is a difference in mortality record of about 24 per cent., to the discredit of the larger litters.

The comparable differences in the alcoholic lines is ever so much greater. There is almost 14 per cent. higher mortality among individuals from litters of two than from litters of one, and actually about 43 per cent. higher mortality among animals from litters of three than from litters of one. The difference between the mortality percentages in the litters of one and the litters of two from alcoholic inbred animals is 50 per cent. In other words, the mortality is three times as high among individuals from litters of two as from litters of one in inbred alcoholics, while the normal individuals born in litters of two are equally as good as those in litters of one. The parents from the injured alcoholic lines are incapable of producing large litters of strong individuals. The subnormal fetus fares pretty well alone in the uterus but is put at a great disadvantage by having to divide its uterine nourishment with brothers and sisters.

Another almost equally plausible explanation of this striking difference in quality and vitality among the members of small and large litters might be given. It may be supposed that the growth capacity of the eggs maturing in the ovaries of normal and subnormal individuals depends somewhat upon the number of eggs maturing at any one time, or ovulation period. A normal animal may be capable of developing two entirely good eggs at an ovulation, or possibly three, whereas a weakened, less vigorous individual has ovaries incapable of producing more than one well-nourished or well-developed egg at any one time. Of course, it is understood that the small size of a mammalian egg would make it seem as though it required very little stored food from the ovary, yet that little must be of an extremely fine quality, since so much of the energy of early development is derived from the materials stored within the egg.

One point which might be interpreted to favor such an explanation is the fact that the small, weak young contained in the large litters do not recover and make their shortage good after birth, as might be expected if their inferior condition was simply due to a lack of nourishment available in the overcrowded uterine environment in which their late stages of development were passed. Lack of intra-ovarian nutrition would certainly produce a more lasting effect, since it occurs at an earlier stage than lack of uterine nutrition, though of course we do not pretend to deny that poor uterine nutrition would also leave its persisting mark.

When only one young was produced in a litter the average growth rate of such individuals during the first month after birth was 85.09 grams. Such specimens were not only largest at birth, but they grew fastest after birth. Animals born in litters of two increased 68.46 grams during the first month after birth, while those born three in a litter gained only an average of 63.6 grams during the same period. In other words, the last group only gained 75 per cent. of the amount gained by similar specimens which were fortunate enough to be developed alone in the ovary and in the uterus.

A second conclusion indicated by Table II is that inbreeding the defective alcoholic stock produces a quality of offspring decidedly inferior to that produced by the alcoholic lines when not inbred. This involves the internal factors of the germ cells. When a modified germ cell is united with a related one probably modified in a closely similar manner, a summation of the modification produces a more decidedly modified individual than would result from the combination of two non-related germ cells, even though they also be modified. In other words, as is shown in much of the data on heredity in higher animals, relatives probably respond to the treatment more nearly in the same way than do non-relatives, and there fore inbred defectives produce the most disastrous results obtainable.

The Relative Conditions of the Male and Female Descendants from Paternal and from Maternal Alcoholized Ancestors

We may now consider the possibility of analyzing the relative influences of various alcoholized ancestors on their offspring of different sex and the descendants of such offspring. The problems may be stated thus: are the offspring from alcoholized males more or less degenerate or modified than those from alcoholized females, and is there a difference in the degree of degeneracy between the male and female offspring? Are the descendants from alcoholic grandparents on the father's side more or less defective than the descendants from alcoholic grandparents on the mother's side, and do alcoholized grandfathers and grandmothers show an equally strong tendency to stamp their grandchildren? Do the grandsons and granddaughters show relatively different conditions, depending upon whether they are descended from alcoholized grandfathers on the father's or the mother's side or from alcoholized grandmothers on the paternal or the maternal side?

Table III, which excludes all inbred animals, is a sum-

TABLE III

THE RELATIVE DEGENERATIVE INFLUENCE OF MALE AND FEMALE ALCOHOLIZED ANCESTORS ON THEIR MALE AND FEMALE DESCENDANTS. (ALL INBRED ANIMALS ARE EXCLUDED)

	Males with	Females with	Young of Unknown Sex with	Males with	Females with	Young of Unknown Sex with	Males with	Females with	Young of Unknown Sex with	Males with	Females with	Young of Unknown Sex with	Males with	Females with	Young of Unknown Sex with	Males with	Females with	Young of Unknown Sex with
		Alcoholized Father			Alcoholized Mother			Alcoholized Grandfather on the Father's Side			Alcoholized Grandfather on the Mother's Side			Alcoholized Grandmother on the Father's Side			Alcoholized Grandmother on the Mother's Side	
Total number..	44	43	70	37	23	38	36	38	26	34	32	48	37	33	36	23	18	25
Lived over 3 months....	37 84.1%	33 76.74%	0	31 91.89%	22 95.65%	0	26 72.22%	23 60.52%	0	23 67.64%	19 59.37%	0	25 67.56%	23 69.69%	0	18 78.25%	14 77.77%	0
		44.58%			57.14%			49%			36.84%			45.28%			48.48%	
Died within 3 months......	7 15.9%	10 23.25%	70 100%	3 8.1%	1 4.34%	38 100%	10 27.77%	15 39.47%	26 100%	11 32.35%	13 40.62%	48 100%	12 32.43%	10 30.30%	36 100%	5 21.74%	4 22.22%	25 100%
		55.41%			42.85%			51.0%			63.15%			54.71%			51.51%	
Deformed.....	1 2.27%	2 4.65%	3 4.28%	0	0	0	3 8.33%	4 10.52%	0	1 2.64%	4 12.5%	7 14.58%	2 5.40%	3 9.03%	7 16.66%	2 8.69%	0	0
		3.82%			0			7.0%			10.52%			10.37%			2.77%	

marized analysis of these questions. The male and female descendants from six different lines are tabulated. The table is not perfectly pure, but merely represents a mass result, since, for instance, in giving the young from alcoholized fathers some of these young had also alcoholized grandparents, etc. The same is true of the other lines. But the large majority of the figures are from unmixed matings, so that these mass results do have some real significance.

In the first vertical section is given the records of offspring from alcoholized fathers. Forty-four males, 43 females and 70 young of unknown sex are considered. Of the males 84 per cent. lived, and 76.7 per cent. of the females lived. These numbers are very high, since in the early part of the experiment only those young which survived were catalogued for sex. Therefore, all of the 70 young of unknown sex were animals which died at birth or soon after, and as the table shows more than half of the animals from alcoholized fathers died soon after birth.

The mortality among the male offspring from alcoholized fathers was 15.9 per cent., while among the female offspring it was considerably higher, being 23.25 per cent. The same difference in quality between the sexes is illustrated by the percentage of gross deformities. Only 2.27 per cent. of the males were deformed, while twice as great a proportion, or 4.65 per cent., of the female offspring from treated fathers were deformed. In all 3.82 per cent. of the offspring from alcoholized males were deformed and the female offspring were inferior in quality to the male.

The next section of the table presents similar data for the offspring from alcoholized mothers. There were 37 male, 23 female and 38 offspring of unknown sex. Again, the offspring in which the sex was ascertained during part of the experiment were only those that survived, therefore, their mortality record is very good, while all the animals of unknown sex were individuals that died shortly after birth. Yet the records of the males and females are based on exactly the same kind of data and are to be fully compared. Eight and one tenth per cent. of the males died, while only 4.34 per cent., proportionately about half as many, females died. Not one grossly deformed animal was found among the offspring of alcoholized females.

Thus from the mortality records the sons of alcoholized mothers appear more affected than their daughters. And taken as a whole the records of the alcoholized mothers are superior in quality to those of the alcoholized fathers, thus indicating that the male germ cells are more injured by the treatment than the female germ cells.

The third section shows the records of the male and female descendants from alcoholized grandfathers on the father's side. Here the mortality record of the males is much better than that of the females; 27.77 per cent. of the males died soon after birth, and 39.47 per cent., a very much higher proportion of the females, died.

The mortality of these animals from alcoholic grandparents seems much greater than that of animals from treated parents; this is due, however, to the fact that the sex of many more of these that died at birth was ascertained as they occurred later in the experiment when this point was being watched. The totals are the only figures in the horizontal mortality columns that are to be compared. The total mortality of descendants from alcoholic grandfathers on the father's side was 51 per cent., which is higher than the mortality of the offspring from alcoholized mothers, 42.85 per cent., but lower than the mortality of offspring from alcoholized fathers, which reached 55.41 per cent.

Among the ascertained male descendants from an alcoholized paternal grandfather 8.33 per cent. showed gross deformities, while 10.52 per cent. of the descendants ascertained to be female were deformed. Considering all the animals in this group, 7 per cent. were deformed, which is almost twice as great a proportion as occurred among the offspring of alcoholized fathers. *The deform-*

ities in the F_2 generation are more frequent than in the F_1.

The fourth section shows the influence on the grandchildren of an alcoholized grandfather on the mother's side. This is the most injurious combination shown. Only 36.84 per cent. of the offspring survive. Of the male descendants of an alcoholized maternal grandfather 32.35 per cent. died soon after birth, while proportionally many more, or 40.62 per cent., of the female descendants died. In all a total of 63.15 per cent. of the descendants from alcoholized maternal grandfathers died, which is the highest mortality record obtained.

Among the grandchildren of alcoholized maternal grandfathers 10.52 per cent. were deformed, a very high proportion. But of the grandsons only 2.64 per cent. were deformed, while almost five times as many, or 12.5 per cent., of the granddaughters were grossly deformed. Thus *the females of the F_2 generation from a treated maternal grandfather are poorer when considered from the standpoint of mortality record and bodily structure than the male F_2's from the same source.*

The fifth line to be considered is that of an alcoholized grandmother on the father's side. The result of this treatment as shown by the grandchildren is very bad, but not quite so bad as from the alcoholized maternal grandfather just discussed.

From alcoholized paternal grandmothers the conditions of 37 grandsons and 33 granddaughters are to be compared. About the same survival record is shown by both sexes: 67.56 per cent. of the male grandchildren lived and 69.69 per cent. of the females lived. Of all the descendants from this combination, including those in which the sex was not determined, only 45.28 per cent. survived, giving a mortality record of 54.71 per cent., considerably better, by almost 10 per cent., than that of animals from an alcoholized maternal grandfather in the preceding section.

A large proportion of the animals from alcoholized paternal grandmothers were deformed, 10.37 per cent. However, only 5.4 per cent. of the grandsons were deformed, while many more, 9.03 per cent., of the granddaughters were deformed and among the young of undetermined sex 16.66 per cent. were deformed.

In the last section the records of descendants from alcoholized grandmothers on the mother's side are given. There were 23 males, 18 females, and 25 young which died with their sex unascertained. Forty-eight and forty-eight hundredths per cent. of the animals lived. The mortality among the males was 21.74 per cent., about the same as that of the females which was 22.22 per cent. The total mortality being 51.51 per cent. From this combination there occurred a low percentage of deformities, confined entirely to the grandsons. *So that 8.69 per cent. of the grandsons from alcoholized maternal grandmothers were deformed, while none of the granddaughters showed any gross structural abnormalities.*

TABLE IV

Mortality During the First Three Months of the Descendants of Known Sex from Alcoholic Animals (Not Inbred)

Treated with Alcohol	Males			Females			All Together		
	Total Number	Died Early	Mortality	Total Number	Died Early	Mortality	Total Number	Died Early	Mortality
Father	44	7	15.90%	43	10	23.25%	87	17	*19.54%*
Mother	37	3	8.10%	23	1	4.34%	60	4	*6.66%*
Grandfather on father's side	36	10	27.77%	38	15	39.47%	74	25	*33.78%*
Grandmother on father's side	37	12	32.43%	33	10	30.30%	70	22	*31.42%*
Grandfather on mother's side	34	11	32.35%	32	13	40.62%	66	24	*36.36%*
Grandmother on mother's side	23	5	21.74%	18	4	22.22%	41	9	*21.95%*

Table IV presents in a more concise manner certain of the figures considered in the foregoing Table III. Only the mortality records of the male and female descendants from different sources and the total mortality of the several groups is shown by the table and thus a ready comparison of the conditions may be made. Among the offspring from alcoholic fathers 15.9 per cent. of the males died and 23.25 per cent. of the females. *The fe-*

male are more injured than the male offspring of treated fathers. The next horizontal line shows that *the offspring from treated mothers are far better than from treated fathers*, having a much lower mortality. *The male germ cell is more affected by the alcohol than the ovum, therefore treated fathers produce poorer offspring than treated mothers.*

The heterogeneous female descendants from an alcoholized paternal grandfather are more affected than the male, 39.47 per cent. mortality to 27.77 per cent.

The male and female descendants from an alcoholized paternal grandmother show about equal conditions, 32.43 per cent. male mortality to 30.3 per cent. female mortality.

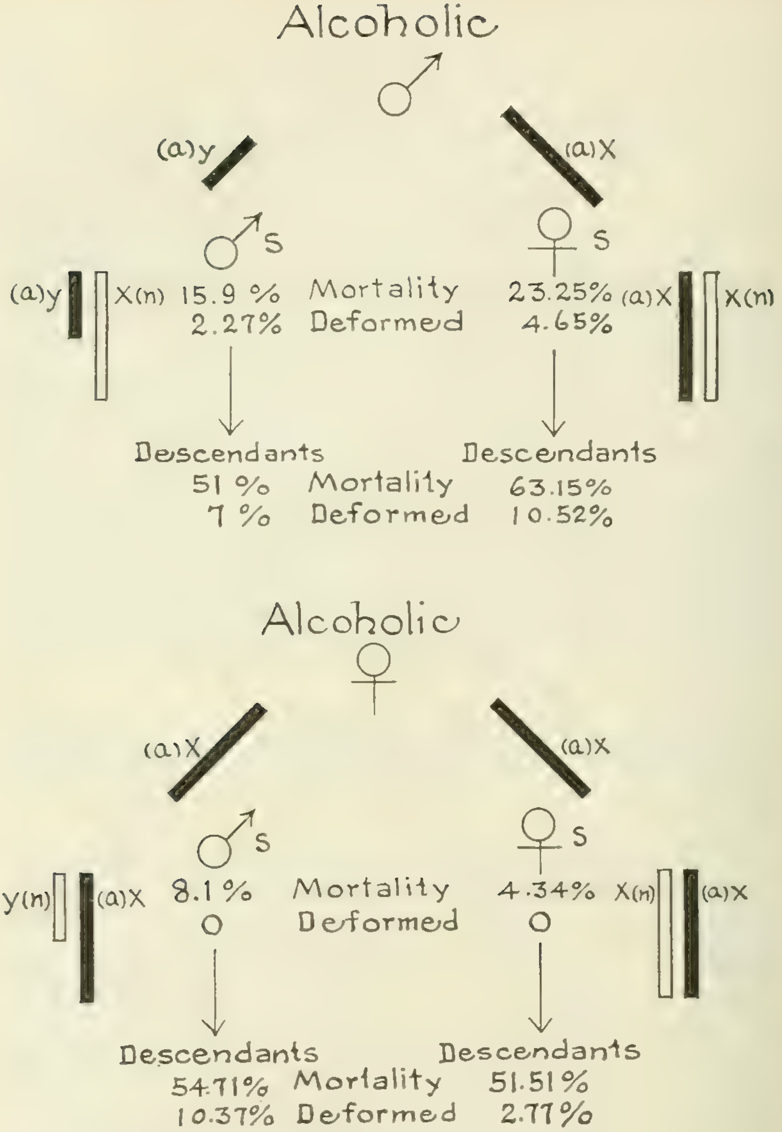
The heterogeneous female descendants are inferior to similar male descendants from an alcoholized maternal grandfather, 40.72 per cent. female mortality to 32.25 per cent. male mortality.

The male and female descendants are about equally strong from an alcoholized maternal grandmother, 21.74 per cent. male mortality to 22.22 per cent. female mortality.

Although explanations of the above differences between the ways in which the male and female guinea pigs are affected by the treatment, as well as explanations of the different records of the grandsons and granddaughters from alcoholization of different ones of the four grandparents, are difficult to give at the present stage of the experiment, a tentative explanation based on the composition of the chromosomal complex is certainly suggested.

GENERAL CONSIDERATIONS

In the case of the male guinea pig, according to the studies of Miss Stevens ('11), two kinds of spermatozoa are produced. The one has a large X chromosome, the female-producing spermatozoon, and the other contains a corresponding small Y chromosome, which from homology with other forms we may consider to be the male-producing. The two classes of spermatids are different



The diagram intended to show the difference in quality between the male and female offspring of alcoholized males and females, and indicating a possible explanation of these differences. The upper part of the diagram represents the heteromorphic chromosomes X and Y of the male passing to the male and female offspring. The larger injured X chromosome gives a larger proportion of deformities and a higher mortality to the females and their descendants. The smaller injured y chromosome gives to the males and their descendants a proportionately smaller amount of deformities and a lower mortality record.

The lower part of the diagram shows equal amounts of injured chromatin passing from the treated mother to her male and female offspring, the black X chromosomes. But these equal amounts of modified chromatin are combined with unequal amounts of normal chromatin from the heteromorphic spermatozoa, the unequal white X and Y chromosomes. The male offspring, therefore, have in their chromosomal constitution proportionally more modified and less normal chromatin than the female offspring which have equal amounts of normal and alcoholic. And the male offspring and their descendants are actually found to show a higher mortality and a greater proportion of deformities than the female offspring and their descendants.

morphologically and we may expect them to differ in their susceptibilities to the alcoholic treatment. One class may be more affected than the other. This might be due simply to the reason that one class of spermatozoa actually according to mass has more chromatin to be acted upon than the other. And this difference in mass of affected chromatin might be sufficient to give a difference in quality between the individuals arising from the two classes of spermatozoa.

At any rate, as the accompanying diagram indicates, there is a decided difference between the records of the male and female offspring from treated guinea pigs. The upper half of the accompanying diagram shows that the mortality is higher and the gross deformities more frequent among the female offspring sired by alcoholized male guinea pigs than among the male offspring. This difference we may venture to suppose is due to the fact that the female offspring actually receive more modified or injured chromatin from the alcoholic father than do the sons. The diagram is an attempt to represent this larger mass of injured chromatin, the large black X chromosome passing to the daughters, while the smaller black Y chromosome is received by the sons.

Another possible explanation might be that the two heteromorphic sex chromosomes, the X and Y, respond differently to the influence of the alcoholic treatment, the X being the more affected. Such an opinion has some basis, since these chromosomes in the later development of the two sexes seem to carry such a number of contrasting qualities according to the splendid evidence presented by Morgan and his associates. One may be permitted to assume on probability, at any rate, that the X and Y chromosomes are qualitatively different in their finer chemical constitutions, and this qualitative difference would necessitate a different response to the chemical treatment on the part of each of the two chromosomes.

There is also important evidence from the parthenogenetic groups, as, for example, the Phylloxerans and

Aphids (Morgan, '09), which might lead one to believe that the two classes of spermatids or finally spermatozoa are never quite equally active or vigorous. This difference may vary from apparent equality in most higher animals to cases such as the parthenogenetic Phylloxerans and Aphids, in which one class of spermatids are actually degenerate and non-functional.

In this connection an experiment performed with a quite different problem in view by Cole and Davis ('14) with alcoholized rabbits is suggestive. They found that when two male rabbits were mated with a female superfetation occurred in most cases so that part of the resulting litter of young were sired by one male and part by the other. The two males differed in their ability, so that one more often sired the majority of young of a given litter and in the total number of competition matings sired the greater number of young. When this male with the fertilizing advantage was treated for a short period of time, a month or more, with fumes of alcohol he was then affected in such a way that when mated in competition with the same male he normally had beaten he now failed to sire any young. Yet if mated singly or alone with a female he still had the power to beget offspring. The alcohol treatment had in some way lowered the power of his spermatozoa to fertilize an egg. Thus these spermatozoa could no longer fertilize an egg in the presence of the spermatozoa from a male which was originally less potent than they.

All of these data indicate differences in the behavior and reactions of the individual germ cells, and such differences probably account for the discrepancy existing between the conditions of the male and female offspring from an alcoholized father. Since this point has only recently been discovered in the experiments, we now have very few definite matings to test its meaning by back crosses with the normal. But a large number of heterogeneous matings have been made during the last few years and their gross results serve to verify the fact that the difference in quality between male and female offspring is actual, although such matings furnish no definite analysis of the conditions.

In the first place, the upper half of the diagram shows that the mortality is higher and the defects more frequent among the female offspring of treated males than among their sons. The products of the heterogeneous matings in which these male and female offspring have taken part go to indicate that the first apparent difference in their records was a real difference. The mortality record of the mass descendants from the sons of alcoholized fathers is about 20 per cent. better than the mortality record from the descendants of the daughters of alcoholic fathers. And the proportion of deformities is 50 per cent. higher among the descendants of the daughters than among the descendants of the sons. These conditions of the descendants prove that the female offspring from the alcoholized males are actually worse than the male offspring in the following respects: their mortality record, the frequency of deformities, and the quality of young to which they give rise. The only plausible way to account for the origin of this difference is to assume that the female-producing spermatozoa were more modified by the treatment than the male-producing spermatozoa. Whether such an increased modification is due to the presence of a greater mass of chromatin to be injured in the one case than in the other or to a difference in response on the part of the two heteromorphic sex chromosomes it is impossible to state. The difference, however, is a fact!

The lower half of the diagram illustrates the different qualities of the male and female offspring from alcoholized mothers. Here each sex of the offspring in accordance with prevalent cytological views receives an equal amount of chromatin from the treated mother. And, moreover, as far as the treated mother is concerned similar chromosomal complexes are conveyed to both sexes of the offspring. The two classes of young should, therefore, show similar conditions, but such is not the case.

The mortality of the male offspring is higher than that of the female. This condition may probably be explained on the same principles we have employed above. The two sexes receive equal amounts of injured chromatin from their alcoholized mother, but this injured chromatin in the case of the female individuals is mixed with a larger amount of normal chromatin from the father than is the case with the male. The female combination of equal amounts of good and bad chromatin gives rise to a better product than the male combination of a larger amount of modified chromatin with a smaller amount of good. Therefore the records of the male offspring are inferior to those of the female offspring.

The female combination, ovum and spermatozoon with equal amounts of chromatin, good and bad, is proportionately less injured than the male combination, ovum with a larger amount of bad and spermatozoon with a smaller amount of normal chromatin. The diagram represents the black X chromosomes equal in size passing to the daughters and the sons to be combined with the large white normal X in the case of the daughters and with the small white normal Y in the case of the sons.

Again the descendants from heterogeneous matings of these males and females prove that there is an actual difference in quality. The descendants from the sons of alcoholic mothers show a slightly higher mortality and a much greater proportion of deformities than are found among the descendants of their daughters.

We believe that these results actually show a difference in response to the treatment on the part of the male- and female-producing spermatozoa. Such a difference logically follows the cytological differences in structure which Wilson and others have so clearly demonstrated during the past ten years. If this structural difference is of any significance, as it surely must be, then such physiological differences in behavior as are indicated in our results should sooner or later be found.

On such a basis as this the sex ratio in different classes of animals may possibly be explained. A species such as man, which constantly seems to produce more males than females, may be said to form more active or vigorous male-producing spermatozoa. In the competition to fertilize the egg such spermatozoa win an advantage and in the sum total more males than females arise, the ratio depending upon the extent of the advantage the one class of spermatozoa has over the other.

We now have under way a number of matings which are designed to test these propositions in an analytical fashion. One of us (Papanicolaou, '15) is in possession of data giving reason to believe that a second explanation may be offered to account for the different conditions presented by the male and female offspring produced by alcoholized females. Such an explanation is based on the supposition that the female guinea pig as well as the male has a share in the determination of the sex ratio and may produce two kinds of ova. Such an explanation in its final analysis is extremely complex and unnecessary in the present discussion, though it will be presented in a future consideration of the regulation of the sex ratio in these animals.

Admitting, as is suggested above, that the two groups of spermatozoa differ in their response and resistance to the treatment, we may also admit that there are other normal differences in their vitality and behavior. These normal differences must also vary within certain limits. In one group of animals the female-producing spermatozoa may be more active and possess a higher degree of fertilizing power than the male-producing spermatozoa. Such a group would show a sex ratio below one hundred, there being more females than males produced. In other species of animals with a sex ratio of more than 100 the reverse condition obtains; the male-producing spermatozoa possess on an average a higher fertilizing power than the female-producing. But the advantage of the male-producing sperm may be slight and no doubt many individual males tend to form female-producing sperm with a higher fertilizing power than the male-producing. Such individuals will more frequently beget female offspring. Slight differences in the physiological behavior of the two classes of spermatozoa would account for the sex ratios in all animals, and finally, as Morgan has shown, the extreme difference between the qualities of the two classes of spermatozoa leads to the degeneration of one entire class and the necessary production of only one sex from the fertilized eggs of these species. Such species must also be parthenogenetic in order to produce individuals of the other sex.

This discovery by Morgan suggests, as Wilson ('11) brings out in his review of the sex chromosome question, a plausible explanation of the sex ratios in different classes of animals. And we believe the evidence presented above lends further support to such an interpretation.

A rather old popular idea in attempting to explain the sex riddle may have some ground of fact from the standpoint of the variations in the differences of fertilizing power of the two classes of spermatozoa. It has often been claimed that one testis is male-producing and the other female-producing. Every one knows that this is untrue. Yet one testis may have a tendency to produce spermatozoa of the female class with a higher fertilizing power than the male sperm of this testis, and the other testis might have an opposite tendency, since the conditions of behavior often differ in two organs of a bilateral pair. An animal which has produced a large proportion of male offspring may after semi-castration produce almost all female offspring. A possible explanation for such an occurrence would be that the removed testis had produced more vigorous male sperm than female and the spermatozoa of this testis possessed the higher fertilizing power, while the remaining testis tended to produce more potent female sperm. On removing the one testis the other came into supremacy. In the same imaginary case, if the opposite testis had been removed, there would have been no change in the tendency to produce offspring of a certain sex, since the remaining testis originally possessed an advantage.

Finally, then, from the above experiments there is no question that the material basis of the hereditary qualities has been injured, since alcoholized males have transmitted the injury to four generations during a period of almost five years. In other words, as stated above, chromatin injured five years ago is now living in the great-grandchildren of the individuals in which it was injured.

Bardeen with the X-ray and Oscar Hertwig with radium have induced similar injuries by directly treating the spermatozoa, but these cells were so greatly injured that only the immediate effect upon the developing embryo was shown. The present experiments, however, demonstrate the passage or transmission of the injured chromatin from generation to generation during a period of years. *The behavior of the carriers of heredity becomes pathological just as any other organ with a normal function may behave in an abnormal or pathological manner.*

Mammals are particularly adapted to the study of such features of heredity as this, on account of their typical structure and large, easily observed organs. The complexity of their structure and behavior further permit the possibility of slight modifications becoming visible through abnormal conditions of their nervous system, etc. Thus with such material as guinea pigs a few experiments of this kind may furnish certain clues to the processes of behavior of the chromosomes that less plastic and simpler forms might never present in such a manner as would be recognizable.

On the other hand, the small litters and comparatively slow breeding render these higher animals unsuitable for an exhaustive analysis of many of the intricate problems of normal heredity.

Summary and Conclusions

In the foregoing pages we have considered the results of an experiment now in progress for more than five years which analyzes to some extent the influence on the offspring of alcoholizing either one or both parents and the manner of hereditary transmission of the induced effects to subsequent generations.

The experiments have demonstrated on two different stocks of normal guinea pigs that the parental germ cells may be so modified by chemical treatments that they are rendered incapable of giving rise to a perfectly normal offspring. This incapacity is probably due to modifications of the chromatin or carriers of the hereditary qualities within the germ cells, since the great-grandchildren, the F_3 generation, from the treated animals are usually more decidedly affected and injured than the immediate offspring (F_1) of the alcoholized animals.

This then becomes a study of the behavior of diseased or pathological chromatin in heredity. Chromatin rendered pathological more than four years ago is still living and has now been passed on to the F_3 generation from the alcoholized great-grandparents. The F_3 animals are almost without exception incapable of reproduction and are in many ways subnormal and degenerate.

Studies of abnormal heredity may possibly furnish a means of analyzing the normal methods of action by which the minute carriers of hereditary qualities contained within the fertilized egg are capable of causing complex developmental and structural changes to reoccur from generation to generation in so wonderfully consistent a manner. Just as the knowledge furnished by studies of experimentally modified embryonic development has supplied valuable data towards a clearer understanding of the normal processes and changes which occur in the developing embryo.

The treatment of adult guinea pigs by an inhalation method with daily doses of alcohol through several years produces little if any noticeable effect upon the organs and tissues of the animal's body. The direct action of alcohol fumes tends to injure the respiratory mucosa and to render the cornea of the eye dull or opaque. These changes, however, do not inconvenience the animals in any perceptible way, and they remain strong and hardy and live as long and actively as the untreated guinea pigs. In spite of their healthy appearance the injurious influence of the alcohol inhalation is very decidedly shown by the quality of offspring to which the treated animals give rise. And the descendants of these offspring are even worse than the F_1 generation when compared with the different generations of control animals produced under identical cage and food conditions.

The males seem to be more injured by the treatment than the females, taking as an index of injury the quality of their offspring and descendants. Stating it differently, the spermatocytes or spermatozoa are more sensitive to the changed chemical condition of the tissues than are the female germ cells.

There is a larger proportion of degenerate, paralytic and grossly deformed individuals descended from the alcoholized males than from the alcoholized females.

The records of 682 offspring produced by 571 matings of animals of various types have been tabulated to show the kinds of litters of young produced and their ability to survive. One hundred and sixty-four matings of alcoholized animals, in which either the father, mother, or both were alcoholic, gave 64, or almost 40 per cent., negative results or early abortions, while only 25 per cent. of the control matings failed to give full-term litters. Of the 100 full-term litters from alcoholic parents 18 per cent. contained stillborn young, and only 50 per cent. of all the matings resulted in living litters. Forty-six per cent. of the individuals in the litters of living young died very soon after birth. In contrast to this record 73 per cent. of the 90 control matings gave living litters and 84 per cent. of the young in these litters survived as normal, healthy animals.

The mating records of the descendants of the alcoholized guinea pigs, although they themselves were not treated with alcohol, compare in some respects even more unfavorably with the control records than does the above data from the directly alcoholized animals.

Of 194 matings of F_1 animals in various combinations 55 have resulted in negative results or early abortions, 18 stillborn litters of 41 young occurred, and 17 per cent. of these stillborn young were deformed. One hundred and twenty-one living litters contained 199 young, but 94 of these died within a few days and almost 15 per cent. of them were deformed, while 105 survived and 7 of these showed eye deformities. Among 126 full-term control young of the same stock not one has been deformed.

The records of the matings of F_2 animals are still worse, higher mortality and more pronounced deformities, while the few F_3 individuals which have survived are generally weak and in many instances appear to be quite sterile even though paired with vigorous, prolific, normal mates.

The structural defects shown by the descendants of alcoholized animals seem to be confined chiefly to the central nervous system and special sense organs. Many of the young animals show gross tremors, paralysis agitans; the hind legs, fore legs or both legs of one side may be paralyzed (Plates I and II). Eye defects are very common, such as opaque cornea, opaque lens, various degrees of monophthalmicum asymmetricum, and finally several cases of complete anophthalmia have occurred, the entire eyeballs, optic nerves and optic chiasma being absent (Figs. 1 to 3 and Plate III).

The quality of individuals from the same parentage varies inversely with the size of the litters in which they are produced. Animals born one in a litter are rather strong, even though derived from very bad alcoholic lines. This difference between the members of small and large litters is also shown by the normal animals, but the difference in quality between members of large and small litters is ever so much greater in the alcoholic lines. There is also some tendency on the part of the alcoholic animals to produce a greater proportion of small litters and this aids somewhat towards the perpetuation of their lines.

Inbreeding tends to emphasize the alcoholic effects. This is probably due to related animals responding to the treatment in closely similar ways on account of the similarity of their constitutions. Inbreeding, as such, may be harmful. But inbreeding added to the alcohol effects produces a much worse condition in the offspring than either inbreeding or alcoholism alone could do.

The data from alcoholized male lines indicate that the *female offspring from alcoholic males are less viable and more frequently deformed than the male offspring. And heterogeneous matings of such male and female offspring further emphasize the same inferiority on the part of the female offspring from treated males.* This is a very significant fact.

The fact that the offspring of one sex differ in quality from those of the opposite sex, and that the female offspring of an alcoholic male are inferior to his male offspring suggests at once a difference between the germ cells concerned in the production of the male and female young. Miss Stevens showed that the spermatocytes of the male guinea pig contained a heteromorphic pair of chromosomes and half of the spermatozoa would be expected to receive one member, the X chromosome, of the heteromorphic pair and one half of the spermatozoa the other member, the Y chromosome, of the heteromorphic pair. We now have two possibilities in explanation of the above facts. In the first place, it may be assumed that the alcohol acts similarly on all of the chromatin to injure it. Thus a mass action would cause the spermatozoa carrying the larger member of the heteromorphic pair to deliver more injured chromatin and the other spermatozoa with a less total amount of injured chromatin would deliver less when they fertilize eggs containing equal amounts of normal chromatin. The fertilized egg giving rise to the female, therefore, contains a greater proportional amount of alcoholic chromatin to normal chromatin than does the egg giving rise to the male. And so the female product is actually more injured than the male.

A second possible explanation of these conditions may be that the X and Y chromosomes themselves respond differently to the treatment, the X being the more sensitive of the two. But in either case the two classes of spermatozoa certainly seem to respond differently to the treatment and this shows a physiological difference in behavior to correspond with the well-known morphological differences so often found between the two groups of spermatids of many animal species.

The data from alcoholic female lines indicates that *the male offspring from alcoholic females are inferior in quality to the female offspring. And heterogeneous matings of such male and female offspring further prove the inferiority on the part of the male offspring from treated mothers.* This is also significant. How can it be put in accord with the above chromosomal explanations for the difference in quality between the female and male young of alcoholized fathers?

If we admit that all of the eggs arising from an alcoholized female guinea pig are homomorphic and contain groups of chromosomes equal in mass, it follows that her male and female offspring receive the same amount of injured chromatin and should be affected by such chromatin to equal degrees. But this is only part of the case, the injured female chromatin is combined with normal chromatin from the normal father when the eggs are fertilized and here the difference arises. The female offspring receives from the normal father a larger amount of normal chromatin than do the male offspring. So that the female arises from an egg in which equal amounts of good and injured chromatin are present, while the male offspring arises from an egg in which a larger amount of injured chromatin is united with a smaller amount of normal. Therefore, proportionally, the male offspring from treated mothers have more injured chromatin in their entire bodily make up than do the female offspring, and are comparatively in a more abnormal condition.

Another explanation of these differences between the male and female offspring of alcoholized females could be based on the possibility of the female being heterozygous for sex. This involves a very complex discussion, but one for which there is some ground on the basis of the regulation of the sex ratio in these animals.

Finally, then, the experiments show the hereditary transmission through several generations of conditions resulting from an artificially induced change in the germ cells of one generation. And they furnish data of importance bearing upon the pathological behavior of the carriers of heredity as well as the differences in behavior between the two types of germ cells produced by an animal carrying heteromorphic chromosomes.

LITERATURE CITED

Bardeen, C. R. 1907. Abnormal Development of Toad Ova Fertilized by Spermatozoa Exposed to the Roentgen Rays. *J. Exp. Zool.*, IV, p. 1.

Cole, L. J., and Davis, C. L. 1914. The Effect of Alcohol on the Male Germ Cells, Studied by Means of Double Matings. *Science*, N. S., 39, p. 476.

Hertwig, O. 1913. Versuche an Tritoneiern über die Einwirkung bestrahlter Samenfäden auf die tierische Entwicklung. *Arch. f. Mikr. Anat.*, 82, Abt. II.

Morgan, T. H. 1909. A Biological and Cytological Study of Sex Determination in Phylloxerans and Aphids. *J. Exp. Zool.*, VII, p. 239.

1912. The Elimination of the Sex Chromosomes from the Male-producing Egg of Phylloxerans. *J. Exp. Zool.*, XII.

1910 to 1915. Numerous Studies on Inheritance in *Drosophila*. *J. Exp. Zool.*, *Biol. Bulletin*, AM. NAT., etc.

Papanicolaou, George. 1915. Sex Determination and Sex Control in Guinea Pigs. *Science*, N. S., 41, p. 401.

Stevens, N. M. 1911. Heterochromosomes in the Guinea Pig. *Biol. Bull.*, XXI, p. 155.

Stockard, C. R. 1910. The Influence of Alcohol and Other Anesthetics on Embryonic Development. *Am. J. Anat.*, 10, p. 369.

1912. An Experimental Study of Racial Degeneration in Mammals Treated with Alcohol. Arch. Internal Med., X.

1913. The Effect on the Offspring of Intoxicating the Male Parent and the Transmission of the Defects to Subsequent Generations. AM. NAT., XLVII, p. 641.

1914. A Study of Further Generations of Mammals from Ancestors Treated with Alcohol. *Proc. Soc. Exp. Biol. and Med.*, XI, p. 136.

1914. The Artificial Production of Eye Abnormalities in the Chick Embryo. *Anat. Record*, 8, p. 33.

Wilson, E. B., 1905–09. Studies on Chromosomes I to V. *Jour. Exp. Zool.*, Vols. 2, 3, and 6.

1911. The Sex Chromosomes. *Arch. Mikr. Anat.*, 77.

1914. Croonian Lecture: The Bearing of Cytological Research on Heredity. *Proc. Royal Soc.*, 88.

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AN EXPERIMENTAL ANALYSIS OF THE ORIGIN AND RELATIONSHIP OF BLOOD CORPUSCLES AND THE LINING CELLS OF VESSELS

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Studies on the origin and development of the cellular elements of the blood and the so-called endothelial cells which line the blood vessels in the normal embryo are peculiarly difficult on account of the important rôle that wandering mesenchyme cells play in these processes. The problem is also further confused by the perplexing mixture of cells of different origin brought about by the early established circulation of the body fluids. The development of no other embryonic tissue is so disturbed by mechanical and physical conditions.

A study of living fish embryos with the high power microscope has made it possible to observe the behavior of the wandering cells and to follow them in their development. The disadvantages due to the intermixture of cells in the blood current have been overcome by the investigation of embryos in which a circulation of the blood is prevented from taking place.

When the eggs of the fish, Fundulus heteroclitus, are treated during early developmental stages with weak solutions of alcohol, the resulting embryos in many cases never establish a blood circulation. In other respects these embryos may be very nearly normal and the development and differentiation of their tissues and organs often proceed in the usual manner, though at a somewhat slower rate. The heart and chief vessels are formed and the blood cells arise and develop in a vigorous fashion. The heart pulsates rhythmically but is unable to propel the body fluid since its venous end does not connect with the yolk vessels. And in many cases its lumen is partially or completely obliterated by periblastic material and nuclei which seem to be sucked into the heart cavity from the surface of the yolk.

In these embryos without a circulation of the blood, one is enabled to study the complete development of the different types of blood corpuscles in the particular regions in which they originate. There is no contamination of the products of a given region through the introduction of foreign cells normally carried in the blood stream.

The actual power to form blood cells possessed by the different organs and tissues may be determined in the experimental embryos having no blood circulation. And the true haematopoetic function is thus clearly contrasted with the ordinary reproduction or multiplication of blood cells which might take place within the tissue spaces of such organs in the normal embryo.

The debated question regarding the production of blood cells from those cells lining the blood vessel wall may be convincingly answered, at least for the species here studied.

The results and conclusions derived from these experiments may be summarized as follows:

1. The fish embryo is capable of living and developing in an almost normal fashion without a circulation of its blood; this fact was first recorded by J. Loeb in 1893, yet this is the initial study of blood and vessel formation in such embryos. Red blood cells may be seen to arise and differentiate in these living embryos in two definite localities; the one within the posterior body region, and the other the blood islands on the yolk.

The blood cells remain confined to their places of origin, yet they attain a typical red color and may persist in an apparently functional condition on the yolk-sac for as long as sixteen to twenty days. The normal embryo becomes free swimming at from twelve to fifteen days, but individuals without a circulation never hatch although they may often live for more than thirty days.

All recent investigators have claimed that there are no blood islands present on the bony fish yolk-sac. Yet the presence of such islands is readily demonstrated in living Fundulus embryos, in the normal as well as in those with no circulation.

2. The plasma or fluid in the embryos which fail to develop a circulation begins to collect at an early time in the body cavities. The pericardium becomes hugely distended with fluid, as well as the lateral coelomic spaces and the Kupffer's vesicle at the posterior end of the embryo. The great distension of the pericardium due to this fluid accumulation pushes the head end of the embryo unusually far away from the surface of the yolk. The heart is thus often stretched into a long straight tube or string leading from the ventral surface of the head through the great pericardial cavity to the anterior yolk surface.

No blood vessels, or very few if any, form on the extreme anterior portion of the yolk-sac, so that the venous end of the heart is never connected with veins. And the heart does not draw fluid into its cavity to be pumped away through the aorta. When the heart cavity does contain fluid it is unable to escape and small floating particles may often be observed rising and falling with the feeble pulsations.

3. The hearts in embryos without a circulation are lined with a definite endocardium, but the myocardium or muscle wall is poorly developed, sometimes consisting of only a single cell layer. Pigmented cells are not present in the wall of the normal heart, but in the experimental hearts these large chromatophores are invariably found. The cavity in many of the hearts is almost if not entirely obliterated by the presence of periblastic material and huge amorphous periblast nuclei.

The conus end of such hearts leads directly to a more or less closed ventral aorta. Portions of the aortic arches are seen as open spaces, and the dorsal aortae are almost invariably seen as typical spaces lined by characteristic embryonic endothelium.

A point of much importance is the fact, that *neither these hearts with their endothelial linings nor any portion of the aortae at any stage of development have ever been seen to contain any form of red blood corpuscle.* Cells of this type are completely absent from the anterior regions of the embryo.

4. Pigment cells normally occur on the Fundulus yolk-sac and arrange themselves along the vascular net so as to map out the yolk-sac vessels in a striking manner. Loeb has thought that this arrangement along the vessel walls was possibly due to the presence of oxygen carried by the corpuscles within the vessels. In the embryos without a yolk-sac circulation the pigment cells arise but rarely become fully expanded so that the usual long branched processes are represented only by short projections; the chromatophore consequently seems much smaller than usual.

The unexpanded pigment cells, however, wander over the yolk-sac and collect in numbers around the plasma filled spaces. The yolk surface of the pericardium and the periphery of the Kupffer's vesicle are often almost covered with pigment. The hearts are during early stages full of plasma and the pigment cells form a sheath around them, while such cells are never present on the normal hearts during the embryonic period.

These facts would seem to indicate that the plasma rather than the blood corpuscles contains the substance which attracts the chromatophores and initiates their arrangement along the normal vascular net of the yolk-sac.

5. A definite mass of cells characteristic of the fish embryo is located in the posterior half of the body between the notochord and the gut and extends well into the tail region. This so-called 'intermediate cell mass' is the intra-embryonic red blood cell anlage in many of the species.

The peripheral cells of the mass as claimed by Swaen and Brachet, or the mesenchyme about the mass, Sobotta, form a vascular endothelium which encloses the central early blood corpuscles. In individuals without a circulation the erythroblasts arise in a normal manner in this centrally located position and become erythrocytes or red blood corpuscles filled with haemoglobin. Typical vascular endothelium completely surrounds the erythrocytes which, instead of being swept away as usual by the circulating current, remain in their place of origin. All of the early blood forming cells of this intermediate mass give rise to red corpuscles and never to white blood cells.

6. Contrary to the opinion of most recent observers on blood development in Teleosts, the Fundulus embryos both with and without a circulation, possess blood islands on the posterior and ventral portions of the yolk-sac. These blood islands are formed by wandering mesenchymal cells which migrate out from the caudal region of the embryo. They represent all that remains on the yolk-sac of the peripheral mesoderm in the Teleosts and probably wander away from mesoderm related to that of the intermediate cell mass. The intermediate cell mass may possibly represent the bulk of the peripheral mesoderm which is here included within the embryonic body, while in other meroblastic eggs it is spread out posteriorly over the yolk. The only mesodermal elements of the yolk-sac in Fundulus are the independent wandering mesenchyme cells, some of which group themselves to form the blood islands, while others give rise to the yolk vessel endothelium, and still other wandering cells develop into the chromatophores.

7. The non-circulating red-blood corpuscles within the embryo remain in a fully developed condition for eight or ten days and then undergo degeneration. In an old embryo of sixteen days it is sometimes found that very few of the corpuscles in the intermediate mass are still present and these are degenerate. The vascular endothelium has been lost and numerous mesenchyme cells have wandered in to lie among the corpuscles.

On the yolk-sac the corpuscles no doubt have a better oxygen supply and here they maintain their color longer, but finally also present a degenerate appearance with small densely staining nuclei and cell bodies much reduced in size.

8. Vascular endothelium arises *in loco* in many parts of the embryonic body other than those localities in which blood cells form. The endothelium is in all cases utterly incapable of giving rise to any type of blood cell. This incapacity cannot be attributed to the abnormal condition of the embryo, since true blood cell anlagen in the same specimen produce blood corpuscles in abundance.

The lining of the vessel walls in the fish embryo has no blood forming function.

9. Neither lymphocytes nor other types of white blood corpuscles, have been found to arise in the yolk-sac blood islands, nor within the intermediate cell mass. The embryonic white blood cells are found most abundantly in the anterior body and head regions, and there occupy extra-vascular positions usually lying among the mesenchymal cells.

The sources of origin of the white and red blood corpuscles in Fundulus embryos are distinct, and these two different types of cells cannot be considered to have a monophyletic origin except in so far as both arise from mesenchymal cells.

The adult blood of Fundulus contains lymphocytes and several varieties of granular leucocytes.

10. There is evidence to indicate that definite environmental conditions are necessary for blood cell proliferation or multiplication. Blood cells do not normally divide when completely enclosed by vascular endothelium. This is the key to the shifting series of so-called haematopoetic organs found during embryonic development.

Erythroblasts, embryonic red blood corpuscles, lying about spaces unenclosed by vascular endothelium, proliferate steadily and give off their products into the space from which they find their way into the embryonic vessels. Should such an erythroblast be carried by the circulation to another unlined space, it may become arrested there and again undergo a series of divisions, giving rise to other erythroblasts. When, however, these spaces become lined by endothelium, the blood cell reproduction stops.

In most embryos the earliest blood cell formation occurs in the yolk-sac blood islands. The cells in these islands continue to divide until they become surrounded by endothelium, then the yolk-sac blood islands lose their haematopoetic function and become a vascular net through which the blood circulates. The liver now takes up the rôle of harboring dividing blood cells within its tissue spaces. When these spaces become vascularized by endothelium, here again the blood cells no longer multiply but merely circulate.

Finally, in the mammalian embryo, one organ after another ceases to offer the necessary harbor for dividing blood cells, until the red bone marrow is the only tissue presenting the proper relationship of spaces and vessels, and here alone the erythropoetic function exists to supply the red blood cells for the entire body circulation. The red blood corpuscles are always produced so as to be delivered into the vessels and thus very soon occupy an intra-vascular position and cease to divide, while the white blood cells arise and remain for some time among the mesenchymal tissue cells in an extra-vascular position.

11. Lymphocytes and leucocytes, so-called white blood cells, along with the invertebrate amoebocytes, are all generalized more or less primitive wandering cells, and are almost universally distributed throughout the metazoa.

Erythrocytes, red blood corpuscles, are very highly specialized cells with a peculiar oxygen carrying function, due to their haemoglobin content. In contrast to the universal distribution of the leucocytes, the erythrocytes, the red corpuscles, are only found in the vertebrate phylum and in a few of the higher invertebrate groups. Yet in these invertebrates the oxygen carrying blood cell never presents the typically uniform appearance of the vertebrate erythrocyte. The oxygen carrying function in many invertebrates is confined to the liquid plasma.

The typical vascular endothelial cell is widely distributed in the animal kingdom and appears to be a simple slightly modified mesenchymal cell.

These three very different types of cells all seem to arise from mesoderm—the mesenchyme. Yet the present investigation would indicate that each arises from a distinctly separate mesenchymal anlage. The erythrocyte, red cell, anlage is localized and perfectly consistent in the quality of its production. The lymphocyte and leucocyte, white cell, anlage is more diffusely arranged and not definitely localized in any particular cells group. The vascular endothelium appears to be formed *in loco* in almost all parts of the embryonic body, and its formation is absolutely independent of a circulating fluid or the presence of blood cells.

On the yolk-sac of Fundulus embryos one finds four distinctly different products, red blood corpuscles, endothelium, and two varieties of chromatophores, differentiating from the apparently similar wandering mesenchymal cells. The environment in which the four types differentiate is identical as far as is possible to determine, and the only explanation of their various modes of differentiation is that the original mesenchymal cells that wandered out were already of four potentially different classes. These differences in potentiality within the cells produced the four different types of structure in one and the same environment. The four types of cells are then in an embryological sense polyphyletic in origin.

Therefore, *vascular endothelium, erythrocytes and leucocytes, although all arise from mesenchyme, are really polyphyletic in origin; that is, each has a different mesenchymal anlage.* To make the meaning absolutely clear, we may consider the origin of the liver and pancreas cells a parallel case, both arise from endoderm but each is formed by a distinctly different endodermal anlage, and if one of these two anlagen is destroyed the other is powerless to replace its product.

A more complete account of this work, with a discussion of the literature bearing on the subject, appears in the September and November numbers of the *American Journal of Anatomy.*

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49. An experimental study of the origin of blood and vascular endothelium in the Teleost embryo.

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The study of the origin and development of the blood and vascular endothelium has proven a difficult problem, mainly on account of the fact that the circulating fluids of the embryo usually begin to flow before the cellular elements of the blood are completely formed. These early developing cells are thus quickly washed from their places of origin and diffusely scattered throughout the embryonic tissues. All types of corpuscles are therefore found in intimate association, whether their origins may have been from a common center or from distinctly separate sources. The study of no other tissue presents this obstacle. It has thus seemed highly advantageous to obtain material in which the circulation of the body fluids might be prevented without seriously altering the normal processes of development.

Several years ago I observed that the eggs of the fish, Fundulus, when treated with weak alcohol, chloroform, ether and other solutions developed embryos in which the blood failed to circulate although the heart pulsated in a feeble manner. During the last three years a systematic study of the origin and development of the blood and vessels in embryos with a heart beat but without a circulation has been conducted. This investigation of the experimental material has at all times been controlled by a study of the blood in the normal embryos.

Such material permits the analysis of the following propositions: Do blood corpuscles and vascular endothelium have a common origin from definite anlagen, or does the one arise from a localized anlage and the other from widely distributed sources? Does vascular endothelium ever give rise to any type of blood corpuscles? Do all types of blood corpuscles arise from a common anlage? Do certain organs such as the liver have a true hematopoietic function or simply serve as a seat for the multiplication of blood cells derived from other sources? What rôle does circulation and function play in the normal development and history of blood corpuscles? Finally, the specific question, is the bony fish an exception to the rule that all eggs with meroblastic cleavage develop blood islands in the yolk-sac? All recent workers claim that there are no blood islands in the Teleost yolk-sac.

In many instances the embryo develops in a fashion closely approximating the normal when the heart beat is fairly strong. The blood fails to circulate, however, on account of the fact that the heart is either blind at one or both ends or fails to connect with the veins. In a normal embryo the plasma begins to flow from the vessels of the embryo out into the sinusoids of the yolk-sac, and there establishes a complex vitelline circulation. In individuals in which there is no circulation the plasma accumulates in the pericardial sinus and also in Kupffer's vesicle at the posterior end of the body.

The distended pericardial vesicle forces the head end away from the yolk sphere and thus stretches the heart out into a long straight thread-like structure. These hearts present a great variety of forms depending upon the extent of the pressure in the pericardium. When studiep in sections such hearts are in some cases actually solid strings of cells, an inner endothelial string surrounded by a single layer of myocardial cells. In other cases they are slender endothelial lined tubes, while in still others the endothelial cavity is distended and filled with plasma although both ends are closed so that in life the plasma is churned up and down by the pulsation of the heart yet is prevented from escaping or flowing out through the aorta.

The dorsal aortae are in certain specimens almost impossible to identify with high power since their lumina are obliterated—while in other specimens the aortae are well formed endothelial tubes. Yet invariably the aortae never contain any trace of blood corpuscles. The yolk vessels and cardinal veins of such embryos are also distinctly lined with vascular endothelium. It must be concluded from abundant observations that the endothelial vessel linings are present and may arise in all parts of the embryo and do not arise from a local anlage situated in some limited part of the embryo or yolk-sac.

The blood of the Teleost has been found to arise from the so-called 'intermediate cell mass'--Swaen and Brachet, Ziegler, Sobotta and others. These authors differ, however, as to the origin of the cells of the 'intermediate cell mass,' claiming them to be separated from the myotomes, schlerotomes or to be an accumulation of mesenchymal cells. This cell mass in the material here studied is always found to be distinctly connected with and derived from the mesenchyme. Early workers claimed that the blood of the Teleost arose in blood islands on the yolk-sac—but more recent investigators have held that the Teleost forms the marked exception to the rule that in all meroblastic types of eggs the blood arises in islands on the yolk-sac. In the Teleost they hold that the entire blood anlage is within the 'intermediate cell mass.' In Fundulus, however, it is found that blood islands do exist in the yolk-sac and continue their development in this position to give rise to well differentiated masses of erythrocytes when there is no circulation. Normal embryos show these islands distinctly in life and when the circulation is established the corpuscles are swept away in the same fashion as those arising from the intermediate cell mass. The erythrocytes in Fundulus embryos have, therefore, two distinct and limited places of origin, first, in the stem vein or conjoined cardinal veins, and, second, from the blood islands of the yolk-sac.

The stem vein may be single or double, separate cardinals. The blood forming portion is posterior, behind the anterior portion of the kidney and extending into the tail. The yolk islands are always on the posterior and ventral yolk surface and do not extend over the anterior surface.

It is clearly shown by these embryos that the vascular endothelium is of almost universal distribution arising from the mesenchyme, while the blood corpuscles arise from a limited area. The vascular endothelium never gives rise to blood cells. So that the heart aorta and vessels of the anterior end of the body, although invariably lined with endothelium do not contain a single corpuscle in embryos of any age. Embryos have been studied up to 20 days old: the normal embryo hatches and is free swimmimg after the 12th day).

The erythroblasts developing from the 'intermediate cell mass' and from the splanchnic layer of mesenchyme in the yolk-sac are not at first surrounded by endothelium. As development proceeds the cells surrounding the mass which are of the ordinary embryonic mesenchymal type differentiate or flatten out to form an endothelial layer surrounding the blood cell mass.

All of the corpuscles arising in the stem vein and yolk-sac develop into erythrocytes. Numerous cells closely resembling poly-morphonuclear and polynuclear leucocytes are found to arise chiefly in the head region and later such cells are found throughout the body. These cells are often very degenerate in appearance and it cannot be definitely stated what their nature actually is. They occur in the tissue and not within the vessels and are abundant in normal embryos as well as those without a circulation.

The further development of the erythroblasts is significant in embryos without a circulation. These cells reproduce rapidly by mitosis and finally give rise to well formed erythrocytes, the cytoplasm of which contains a normal amount of haemoglobin. The haemoglobin forms in the cells within the stem vein and in the blood islands and attains a bright red color in life. As the embryo grows in size the erythrocytes within the stem vein are further removed from the surface supply of oxygen and after about the eighth day of development they begin to degenerate, and in embryos of sixteen days only a few cells of the erythrocyte type are present in the stem vein along with numerous more or less degenerate mesenchymal cells. The blood islands are better supplied with oxygen and the erythrocytes persist and present a bright red color. When compared with the erythrocytes of a normal embryo those of the blood islands in an old noncirculating specimen show an interesting condition, instead of the healthy, slightly granular nucleus of the fish corpuscle the nucleus is more compact and darkly stained resembling in a striking way the reptilian type of corpuscle or 'Sauroid type' of Minot's classification.

Circulation and normal function seem therefore necessary in order to maintain the typical appearance and structure of the red blood cells in these fish, although such cells originally attain a perfectly typical structure without having circulated.

Finally, these embryos in which the blood cells arise in a normal manner, yet are never permitted to circulate, furnish material for answering in a conclusive way the long contested question—whether the so-called hematopoietic organs such as the liver do actually contain cells which give rise to blood cells, or serve merely to harbor multiplying blood cells in their sinusoids. An examination of the liver of a normal Fundulus embryo of seven or eight days shows it to be very vascular and numerous erythroblasts in mitotic division are often seen. The organ presents the usual hematopoietic appearance.

A similar examination of the liver at any stage of development of an embryo in which the blood has not circulated shows a marked contrast to the normal. The organ is perfectly compact scarcely a vessel is to be found with the highest power on thin sections. *Such a liver does not contain a single erythroblast or erythrocyte in any condition* The liver of the bony fish has no true hematopoietic function.

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DIFFERENTIATION OF WANDERING MESENCHYMAL CELLS IN THE LIVING YOLK-SAC

THE yolk-sac of the teleost egg is a particularly favorable object for observing the movements and migrations of cells in the developing embryo. Such a yolk-sac has only one really definite continuous membranous cell layer, the ectoderm; a true endodermal layer is absent, though a superficial syncytium, the periblast, fuses with the actual yolk surface. The mesodermal layer is represented by numerous separate wandering mesenchymal cells. These freely wandering mesenchymal cells may be clearly observed through the perfectly transparent ectoderm as they move over the surface of the periblast.

The writer has attempted a detailed study of the movements of the mesenchyme cells and their manner of development and differentiation on the yolk-sac. Observations have been made on the normal embryos from the earliest stages at which the mesenchyme wanders out upon the yolk up to the late embryo in which a complex vitelline circulation is fully established, and all of the products of the yolk mesenchyme completely differentiated. The study has been greatly facilitated by a comparison of the normal embryos with specimens in which the circulation of the blood was experimentally prevented from taking place. In such specimens the cells on the yolk-sac never became confused or contaminated with other cellular elements introduced by the circulating blood. The wandering cells may thus be completely followed through all stages in their isolated position.

In the first place, I can not resist the impulse to highly recommend that all students of hematogenesis spend some time at least in a study of living mesenchymal cells and their histogenesis. Such a study will soon convince one of the great disadvantages under which an investigator labors in attempting to solve the origin of blood from observations on dead material in serial sections. The problem becomes so simplified and devoid of laborious uninstructive technique that it seems almost superficial. One may learn as much from the living yolk-sac in an hour of careful study as in almost a week's perusal of sections. Most important is the fact that certain things may actually be seen to occur that sections could scarcely stimulate the mind to imagine. The only disadvanage is that the worker may be led to wonder whether so apparently simple a problem is actually of scientific importance. Fortunately, this mental state is soon passed over on realizing the necessary care and precaution which must be taken in following the movements and changes in the living cells.

Each cell is to be recognized as a living complex and the observer will realize the importance as well as the difficulties of thoroughly understanding and interpreting correctly its manifold changes and behavior. Material which to some extent allows such a study is often available. The *Fundulus* yolk-sac, however, is exceptionally adapted to this study on account of the beautiful simplicity of its structure, as well as the remarkable clearness with which each cell may be observed.

The results of this investigation of wandering mesenchymal cells may be summarized as follows:

The wandering cells begin to migrate away from the embryonic shield or line of the embryonic body at an early period, when the embryo is about forty hours old, the germ ring having almost completely passed over the yolk sphere to enclose its vegetal pole. The cells migrate away chiefly from the caudal end of the embryo, only a few wandering out from the head region. The regions of the yolk-sac thus suggest an area opaca about the tail end and an area pellucida around the neighborhood of the head.

All of the cells wander into the so-called subgerminal cavity, the space Wilson[1] and others consider a late stage of the segmentation cavity, between the yolk-sac ectoderm and the periblast syncytium.

When the cells first appear they are all closely similar in shape and about the same size. Very soon, however, they begin to exhibit certain differences. Many become elongate spindle cells with delicate filamentous processes, sometimes producing a stellate appearance. Others are more ameboid in shape with conical pseudopod-like processes which are constantly being thrown out at one place and withdrawn at another. Still a third class of cells appears somewhat later than the other two; these are more circular in outline with short pseudopods and are more slowly moving.

The movements of these extremely numerous cells and their changes of position may be readily followed with a high magnification. In embryos of about sixty hours, still some time before the heart begins to beat or the blood to flow, four clearly distinct types of cells can be recognized among these originally similar mesenchymal cells, and the further history of the four types may be completely traced.

The ameboid cells with conical pseudopod-like processes shortly after sixty hours begin to show an accumulation of pigment granules within their cytoplasm. Just at this time they are seen to be of two distinct varieties,

[1] Wilson, H. V., "The Embryology of the Sea Bass (*Serranus atrarius*)," *Bull. U. S. Fish Com.*, 1891.

one depositing a black and the other a brownish-red pigment.

The black chromatophore increases rapidly in size and by the end of the third day becomes an enormous ameboid body wandering over the yolk. These cells are attracted to the walls of blood vessels and plasma-filled spaces, such as the pericardial cavity becomes in individuals without a blood circulation. When the embryo is five days old the chromatophores are abundantly arranged along the walls of the vitelline vessels, but the pigmented cells are distinctly separate. After this time neighboring cells begin to fuse along their adjacent borders and large pigment syncytia are formed which completely surround and ensheath the vessels. A single syncytium is often of considerable extent.

The brown chromatophores have a somewhat different history. They never become so massive as the black, and their processes are more delicate and graceful in appearance. Yet these cells also attain a large size and in embryos of 72 hours are scattered over the entire yolk-surface. After the third day when the blood begins to flow in the yolk vessels, the brown chromatophores likewise become attracted to the vessel wall. These exquisitely branched cells apply themselves to the wall of the vessel and may often completely surround it. This type of chromatophore, however, always maintains its cellular individuality and never fuses with other cells to form a syncytium, as is the case with the black type.

The function of the chromatophores on the yolk-sac is most difficult to decide, but one thing is certain, they never become changed into any type of blood cell. The brown chromatophore in early stages may accidentally reach the blood current; it then becomes spherical and may readily be observed for a long time on account of its huge size as compared with the blood cells. It never, however, changes in type.

In specimens without a circulation of the blood both types of chromatophores arise in a normal manner and differentiate normally. Their arrangement along the vessel walls fails to occur. The chromatophores, therefore, remain scattered over the yolk or collected about the plasma filled spaces. The heart in such embryos is sheathed with pigment, while the normal heart never has a chromatophore on it.

The elongate spindle cells with their delicate filamentous processes are small in comparison with the two chromatophore types. These spindle cells retain in general their original appearance, but their behavior is most important. In embryos of about forty-eight hours such cells aggregate into certain rather definite groups; later, the groups become more linear in shape and finally these lines of cells arrange themselves so as to form tubular vessels. Several of the larger vessels arise independently upon the yolk, and certain ones of them later become connected with the venous end of the heart, while in all cases capillary nets which also arise independently become connected with the larger vessels. These processes may actually be followed through every step in the living yolk-sac.

The wall of the early vessels is very irregular, with spaces existing between the component cells. Corpuscles are often caught in these spaces or are entangled in the filamentous processes of the endothelial cells. Such conditions in sections would appear as though the corpuscles actually formed a part of the endothelial wall and might incorrectly be interpreted as endothelial cells changing into blood cells. Nothing has been seen in the living embryos to indicate that an endothelial cell has the power to produce a blood cell or to change into a blood cell of any type, but much has been seen to the contrary.

The generalization strikingly made by Thoma[2] that larger vessels arise from a network of capillaries is not true for the large vitelline vessels of the fish yolk-sac. In the specimens without a circulation of the blood the vessels arise and increase in size and persist for a long time without ever experiencing any effect of the blood current upon their walls. In many embryos the circulation after having begun may stop for a time and then later be reestablished, the vessels having persisted in a normal condition. Thoma's so-called laws of vessel formation are, therefore, rudely violated by the development of the vascular system in these embryos.

The vessels arising from independent mesenchymal cells in the space of the blastocele in the teleost yolk-sac entirely overthrow any notion that vessels arise ontogenetically as portions of the celomic epithelium. The vascular lumen is originally continuous with the primary body cavity, the segmentation cavity, and never with the secondary body cavity, or celomic cavity.

The fourth class of cells wander out from the embryonic body somewhat later than the three former types. These are small circular cells with short pseudopod-like processes. They move very slowly, but finally collect into groups on the posterior and ventral regions of the yolk-sphere.

The round cells wander away only from the caudal region of the embryo and probably are derived from the so-called intermediate cell mass which is the anlage of the red blood corpuscles in the fish embryo.

The groups of round cells are slow in their differentiation but just before the circulation of the blood begins, they are seen to be circular erythroblasts. The observer may follow the disappearance of the islands of cells one by one as they are enclosed by the vessels and swept into the circulating stream. About the fifth day these circular erythroblasts become flattened ellipsoidal erythrocytes filled with hemoglobin, the typical red blood corpuscle. The complete change from wandering, more or less globular mesenchymal cells into typical hemoglobin-bearing corpuscles may be followed in the living yolk-sac.

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[2] Thoma, R., "Untersuchungen ueber die Histogenese und Histomechanik des Gefässsystems," Stuttgart, 1893, and "Text-Book of General Pathology and Pathological Anatomy," trans. by Bruce, London, 1896.

In several instances the body proper of the embryo failed to develop or else degenerated very early, yet the yolk-sac formed or persisted with numerous blood islands fully differentiated.

The embryos in which there has been no circulation of the blood form the blood islands from the wandering cells on the yolk-sac, and the constituent elements of these islands differentiate perfectly and may maintain their red color for many days. Yet they never leave the locality in which they have differentiated. The fully formed red blood corpuscles have little if any power of migrating. When the observer can be positive that the blood has never circulated, and this requires very consistent watching, the blood islands of the yolk-sac are always limited to certain regions, and never occur so far anteriorly on the ventral surface of the yolk as to reach the venous end of the heart.

Finally, we may consider the study of the developmental products of the early wandering mesenchymal cells on the yolk-sac of the *Fundulus* embryo as a problem of cell lineage followed to its ultimate end. The primordial mesoderm cell or cells carry within their bodies all the potentialities of the mesoderm and may give rise to a series of cells which are capable of developing muscle, cartilage, bone, connective tissue proper, blood cells, vessels, etc. Yet after a few cell generations the individuals in the series derived from these early cells containing all the mesodermal potentialities no doubt become somewhat limited as to their potentialities. In a certain generation there may be definite cells more or less generally distributed which possess the capacity to give rise to muscle cells, but to no other type of mesodermal tissues. Still later in development these cells may become even more limited in their developmental capacities and thus have the power to produce only a certain type of muscle cell and no other type.

Collections of such cells would then be designated embryologically as the anlage of striated muscle, smooth muscle or heart muscle, as the case might be. Yet it is not to be forgotten that at this stage there might be really no means of distinguishing between the several different types of mesodermal cells.

Limitization of potentialities in the individual mesenchymal cells has apparently reached a comparable stage just about the time when the cells begin to wander upon the yolk-sac of *Fundulus*. We have seen these cells as they wander out and have noted how very soon they may be separated into four distinctly different types, and following the development and behavior of these types it has seemed evident that they are entirely separate and do not intergrade or transmutate. The black chromatophore does not change its nature or divide off other cells which become different in type from the parent cell. Neither do the endothelial cells lining the vessel walls change into chromatophores or into erythroblasts, or vice versa.

From the observations on these yolk-sacs we must conclude that the four types of cells described above have developed from four differ-

ent anlagen, although these anlagen were not necessarily localized groups of cells, but were diffusely scattered mesenchymal cells capable of developing into a definite product, either normal or abnormal, depending upon the nature of the developmental environment. Therefore, the four distinct mesenchymal anlagen each gives rise to a perfectly typical and distinct cell type, although all develop in, as far as is possible to judge, an identical environment, the cavity of the yolk-sac between the ectoderm and the periblastic syncytium. The differences among the four cell types produced are from the standpoint of our present knowledge in all probability due to the potential differences among the apparently similar mesenchymal cells from which they arose. The four types including endothelial cells and erythrocytes we must consider, from an embryological standpoint, as being polyphyletic in origin.
C. R. STOCKARD

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84 (901)

A study of further generations of mammals from ancestors treated with alcohol.

By Charles R. Stockard.

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Experiments now in progress for almost four years have demonstrated the fact that the germ cells of male guinea-pigs can be so injured by allowing the animals to inhale the fumes of alcohol that they give rise to defective offspring although mated with vigorous untreated females.[1]

In the present communication I wish to emphasize the fact that the effect of this injury of the germ cells is not only shown by the immediate offspring of alcoholized animals but is conveyed through their descendants for at least three generations.

The offspring from the treated guinea-pigs which reach maturity are usually nervous and slightly under sized. These animals F_1, or the second generation, are never themselves subjected to the fume treatment. Mated even with normal animals the results are poor when compared with the outcome of normal control matings. Twenty-six matings of second generation with normal animals gave in 4 cases negative results or early abortions, 2 stillborn litters of 4 individuals and 20 living litters containing 31 young, 19 of which died and only 12 survived. Twenty-two matings of second generation by alcoholized animals gave 5 negative results or early abortions, 3 stillborn litters of 7 young and only 14 living liters consisting of 25 young, 11 of which died and 14 survived.

If second generation animals, non-relatives in most cases, are mated with one another the sum total of the result is worse than when they are mated in either of the above combinations. Forty-seven such matings gave in 14 cases negative results or early abortions, 3 stillborn litters containing 8 young, and only 30, less than 64 per cent., living litters consisting of 46 young; 14, or about 33 per cent., died soon after birth and 32 survived.

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[1] *Archiv f. Entw.-Mech.*, XXXV, 1912; *Archives of Int. Med.*, X, 1912; *American Nat.*, XLVII, 1913.

A point of interest is that several of these offspring, F_2, or third generation, show gross defects or deformities. Only two of the immediate offspring from alcoholized animals have shown a clouded condition of the cornea of one eye, and no defect or deformity of any nature has been observed in 89 control young. However, among the 54 F_2, or third generation, young that have reached term 9 or about 17 per cent. show gross eye defects. Two had opaque corneas, 3 complete cataracts in both eyes, the lens being milk white, 2 had one normal eye while the other was about half size and blind, one has only one eye, that of the opposite side being completely absent, and finally one animal was entirely eyeless, having no indication of eyeballs, optic nerves or chiasma.

These abnormal animals arose from parents that had not been subjected to the alcohol treatment, although in all cases two or more of their grandparents, and usually only the paternal ones had been treated for various lengths of time with the fumes of alcohol.

Only a few matings of the third generation, F_2, animals have been made, yet if conclusions may be based upon these small numbers the outcome is more unfavorable than from second generation matings. Six such matings gave in 2 cases negative results or early abortions, 1 litter of 2 stillborn young and 3 living litters containing 5 animals, only 1 of which survived, 4 dying soon after birth.

Two of the four were completely eyeless, the eyeballs, optic nerves, and chiasma being absent. There was an abortive attempt at eyelid formation, a well-formed lachrymal gland and the extrinsic eye muscles were present. These defects no doubt result from injury inflicted upon the germ cells by the experimental treatment. The parents of the anophthalmic guinea-pigs just mentioned were untreated, the four grandparents were also untreated but their great-grandfathers were all alcoholized and their great-grandmothers were all normal animals. It thus appears that the injury received by the germ cells of the great-grandfathers was responsible for the defective condition of their descendants. Many of the defective young have normal maternal ancestry and alcoholized paternal ancestors, the reverse being also true.

Although the descendants of alcoholized males seem to transmit the defects through subsequent generations even more decidedly than the offspring of treated females, yet it is peculiar to find that in pairing F_1, second generation, animals with normal mates if the male of the pair be F_1 the resulting offspring are better and more vigorous than when an F_1 female is mated with a normal male. Eleven matings of F_1 males with normal females gave 1 negative result, 1 stillborn litter of 2 young and 9 living litters of 11 young, 9 of which survived but later gave rise to defective descendants. Fifteen matings of F_1 females with normal males gave 3 negative results, 1 stillborn litter of 2 young and 11 living litters containing 20 young, only 3 of which survived; a result many times more disastrous than that derived from the previous combination.

Since the direct action of the alcohol fumes on the cornea of the eye finally renders many of the treated animals blind one might imagine some connection between this and the defective eye condition of the offspring and their descendants, yet such is certainly not the case. The defective eyes of the descendants of treated animals are due to a generally weakened or impaired development. The male germ cells are weakened or injured by the alcohol treatment and all individuals arising from combinations involving such a germ cell are below normal. In this connection Cole and Davis[1] have recently recorded an interesting experiment with rabbits. They sought to control the experiments on the effects of alcoholic inhalations in a very ingenious way. "By breeding a male homozygous for color and an albino male both to an albino female it is possible to assign the young to their respective fathers, since the offspring of the colored male will be colored and those of the albino male will be albinos. If one of the males now be alcoholized while the other is normal, and offspring from both result, any differences, such as defects in the offspring, may safely be attributed to the effects of the alcoholizing of the male, since both sets of fetuses have developed in the same uterus at the same time, and consequently there can be no question of different environmental influences."

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[1] *Science*, N. S., XXXIX, 1914.

A preliminary test of 36 double matings was made in which both males were normal. One pigmented male was used in 23 of these matings, an albino male also being used in each case—190 offspring were produced and the albino male sired only 24 of them, 166 coming from the pigmented sire. This showed a strong individual potency for the colored male. Yet after he had been alcoholized, by the inhalation method, *he failed to sire any offspring at all* when used in conjunction with an albino male, although he was bred to the female first in at least 5 of the 7 matings made. When bred alone to normal females he sired several litters of young which later showed certain indications of defects. These experiments demonstrate conclusively that the spermatozoön is actually weakened or disabled by the alcohol treatment, as I had formerly concluded in explaining the defective offspring from alcoholized male guinea-pigs.

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56. *Experimental modification of the chromatin within the germ cells of one generation and the resulting hereditary transmission of degeneracy and deformities.* Lantern. Charles R. Stockard, Cornell Medical School, New York City.

The results of an experiment now in progress for more than five years permits to some extent an analysis of the influence on the offspring of alcoholizing either one or both parents and the manner of hereditary transmission of the induced effects to subsequent generations.

The experiments have demonstrated on two different stocks of normal guinea-pigs that the parental germ cells may be so modified by chemical treatments that they are rendered incapable of giving rise to a perfectly normal offspring. This incapacity is probably due to modifications of the chromatin, or carriers of the hereditary qualities within the germ cells since the great-grandchildren, the F_3 generation, from the treated animals are usually more decidedly affected and injured than the immediate offspring (F_1) of the alcoholized animals.

This then becomes a study of the behavior of diseased or pathological chromatin in heredity. Chromatin rendered pathological more than four years ago is still living and has now been passed on to the F_3 generation from the alcoholized great-grandparents. The F_3 animals are almost without exception incapable of reproduction and are in many ways subnormal and degenerate.

Studies of abnormal heredity may possibly furnish a means of analyzing the normal methods of action by which the minute carriers of hereditary qualities contained within the fertilized egg are capable of causing the complex developmental and structural changes to reoccur from generation to generation in so wonderfully consistent a manner. Just as the knowledge furnished by studies of experimentally modified embryonic development has supplied valuable data towards a clearer understanding of the normal processes and changes which occur in the developing embryo.

The treatment of adult guinea-pigs by an inhalation method with daily doses of alcohol through several years produced little if any noticeable effect upon the organs and tissues of the animals' body. The direct action of alcohol fumes tends to injure the mucosa of the respiratory tract and to render the cornea of the eye dull or opaque. These changes, however, do not inconvenience the animals in any perceptible way, and they remain strong and hardy and live as long and actively as the untreated guinea-pigs. In spite of their healthy appearance the injurious influence of the alcohol inhalation is very decidedly shown by the quality of offspring to which the treated guinea-pigs give rise and the descendants of these offspring are even worse than the F_1 generation when compared with the different generations of control animals produced under identical cage and food conditions.

The males seem to be more injured by the treatment than the females taking as an index of injury the quality of their offspring and descendants. Stating it differently the spermatocytes or spermatozoa are more sensitive to the changed chemical condition of the tissues than are the female germ cells.

There is a larger proportion of degenerate, paralytic and grossly deformed individuals descended from the alcoholized males than from the alcoholized females.

The records of 667 offspring produced by 566 matings of animals of various types have been tabulated to show the kinds of litters of young produced and their ability to survive. One hundred and sixty-four matings of alcholized animals in which either the father, mother, or both were alcoholic gave 64, or almost 40 per cent negative results, or early abortions, while only 25 per cent of the control matings failed to give full term litters. Of the 100 full term litters from alcoholic parents 18 per cent contained still-born young, and only 50 per cent of all the matings resulted in living litters, and 46 per cent of the individuals in the litters of living young died very soon after birth. In contrast to this record 72 per cent of the 86 control matings gave living litters and 84 per cent of the young in these litters survived as normal healthy animals.

The mating records of the descendants of the alcoholized guinea-pigs although they themselves were not treated with alcohol compares in some respects even more unfavorably with the control records than did the above records of the directly alcoholized animals.

Of 194 matings of F_1 animals in various combinations 55 have resulted in negative results, or early abortions, 18 still-born litters of 41 young occurred, and 17 per cent of these still-born young were deformed. One hundred and twenty-one living litters contained 199 young, but 94 of these died within a few days and almost 15 per cent of them were deformed, while 105 survived and 7 of these showed eye deformities. Among 115 full-term control young of the same stock not one has been deformed.

The records of the matings of F_2 animals are still worse, higher mortality and more pronounced deformities. While the few F_2 individuals which have survived are generally weak and in many instances appear to be quite sterile even though paired with vigorous prolific normal mates.

The structural defects seem to be confined chiefly to the central nervous system and special sense organs. Many of the young animals show gross tremors, paralysis agitans, or the hind legs, fore legs or both legs of one side may be paralyzed. Eye defects are very common such as opaque cornea; opaque lens, various degrees of monophthalmica asymmetrica and finally several cases of complete anophthalmia have occurred the entire eye ball, optic nerve and optic chiasma being absent.

The quality of the individuals from the same parentage varies inversely with the size of the litters in which they are produced. Animals born one in a litter are rather strong even though derived from very bad alcoholic lines. This difference between the members of small and large litters is also shown by the normal animals but the differences between members of large and small litters is ever so much greater in the alcoholic lines.

Inbreeding tends to emphasize the alcoholic effects. This is probably due to the related animals responding to the treatment in closely similar ways on account of the similarity of their constitutions. Inbreeding as such, may be harmful. But inbreeding added to the alcohol effects produces a much more serious condition in the offspring than either inbreeding or alcoholism alone could do.

The data from alcoholized male lines indicates that the *female offspring from alcoholic males are less viable and more frequently deformed than the male offspring. And heterogeneous matings of such male and female offspring further emphasizes the same inferiority on the part of the female offspring from treated males.* This is a very significant fact.

The fact that the offspring of one sex differs in quality from those of the opposite sex and that the female offspring of an alcoholic male are inferior to his male offspring suggests at once a difference between the germ cells concerned in the production of the male and female young. Miss Stevens showed that the spermatocytes of the male guinea-pig contained a heteromorphic pair of chromosomes and half of the spermatozoa would be expected to receive one member, the x chromosome, of the heteromorphic pair and one-half of the spermatozoa the other member, the y chromosomes, of the heteromorphic pair. We now have two possibilities in explanation of the above facts. In the first place, it may be assumed that the alcohol acts similarly on all of the chromatin to injure it. Thus a mass action would cause the spermatozoa carrying the larger member of the heteromorphic pair to deliver more injured chromatin and the other spermatozoa with a less total amount of injured chromatin would deliver less when they fertilize eggs containing equal amounts of normal chromatin. The fertilized egg giving rise to the female, therefore, contains a greater proportional amount of alcoholic chromatin to normal chromatin than does the egg giving rise to the male, and so the female product is actually more injured than the male.

A second possible explanation of these conditions may be that the x and y chromosomes themselves respond differently to the treatment, the x being the more sensitive of the two. But in either case the two classes of spermatozoa certainly seem to respond differently to the treatment and this shows a physiological difference in behavior to correspond with the well known morphological differences so often shown between the two groups of spermatids of many animal species.

The data from alcoholic female lines indicates that *the male offspring from alcoholic females are inferior in quality to the female offspring. And heterogeneous matings of such male and female offspring further prove the inferiority on the part of the male offspring from treated females.* This is also significant. How can it be put in accord with the above chromosomal explanations for the difference in quality between the female and male young of alcoholized fathers?

If we admit that all of the eggs arising from an alcoholized female guinea-pig are homomorphic and contain groups of chromosomes equal in mass, it follows that her male and female offspring receive the same amount of injured chromatin and should be affected by such chromatin to equal degrees. But this is only part of the case, the injured female chromatin is combined with normal chromatin from the normal father when the eggs are fertilized and here the difference arises. The female offspring receives from the normal father a larger amount of normal chromatin than do the male offspring. So that the female arises from an egg in which equal amounts of good and injured chromatin are present while the male offspring arises from an egg in which a larger amount of injured chromatin is united with a smaller amount of normal. Therefore, proportionally the male offspring has more injured chromatin in his entire bodily make-up than does the female and is comparatively in a more abnormal condition.

Another explanation of these differences between the male and female offspring of alcoholized females could be based on the possibility of the female being heterozygous for sex. This involves a very complex discussion but one for which there is some ground on the basis of the regulation of the sex ratio in these animals.

Finally then, the experiments show the hereditary transmission through several generations of conditions resulting from an artificially induced change in the germ cells of one generation. And they furnish data of importance bearing upon the pathological behavior of the carriers of heredity as well as the differences in behavior between the two types of germ cells produced by an animal carrying heteromorphic chromosomes.

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FEEDING EXPERIMENTS ON RATS

III. A FURTHER CONTRIBUTION TO THE KNOWLEDGE OF ORGANS WITH AN INTERNAL SECRETION

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A series of experiments is being carried on in this laboratory to study the influence upon growth and development of the various internally secreting glands. These glands are being fed to white rats in stated portions, at regular intervals.

The experiments have not proceeded far enough to make a detailed report on the effect of all the organs used. Here merely a preliminary account of some observations on the thyroid treated animals will be given.

Fresh beef thyroid was used and given in portions small enough to keep the animals in fairly good health. In the earlier experiments too large a dose of thyroid had been given, 5 g a week, so that the animals very soon showed all the well-known signs of hyperthyroidism usually leading to death. The dose was gradually cut down to 1 g a week, in other experiments to 1 g in 5 days. Even with such small doses the symptoms of hyperthyroidism were sometimes slightly noticeable, however, the animals kept so well that they were able to produce offspring. After the dose of thyroid had been cut down considerably, the animals not only bred, but the young were also strong enough to keep alive.

In the following list four matings will be described in detail, the offspring of which are still living.

I. ♂*t* × ♀*t*[1]

HISTORY OF FATHER

Born July 21, 1913.
Thyroid feeding began January 6, 1914.

1. Mating: ♂*t* × ♀*t* 1/26–2/16[2] 1914; died; no pregnancy.
2. Mating: ♂*t* × ♀*t* 2/19–3/10, 1914; ♀ died; no pregnancy.
3. Mating: ♂*t* × ♀*t* 3/21–5/15, 1914; no pregnancy.
   Thyroid feeding stopped 5/15, 1914.
4. Mating: ♂*t* × ♀*n* 5/15–6/8, 1914. 10 young 6/10. Frail, died within a week.

HISTORY OF MOTHER

Born December 31, 1914.
Thyroid feeding began 3/24, 1914.
Thyroid feeding stopped 6/10, 1914.

Final mating which gave living offspring

♂*t* × ♀*t* 6/12–7/6, 1914. 3 young born 7/9, 1914. Young are living now. They are much smaller (figs. 1, 2) than the normal rats of the same age (fig. 3). At the age of 152 days they weighed 97 g, 101 g, 190 g resp. Normal rats of this age weigh from 150–220 g.

SUMMARY OF CASE I

(a) While under treatment the father was bred unsuccessfully to 3 treated ♀.

(b) After discontinuation of the thyroid treatment the father was bred to a non-treated ♀. 10 young were born after 26 days, all so frail that they died within a week.

(c) One month after the thyroid treatment had stopped, the father was bred to the mother, the thyroid treatment of which ceased on the mating day. The results were 3 undersized young born 54 days after the father and 29 days after the mother had received the last dose of thyroid.

In this case father and mother received thyroid *before* mating.

II. ♂*t* × ♀*n*.

HISTORY OF FATHER

Born January 28, 1914.
Thyroid feeding began 3/24, 1914.
Thyroid feeding stopped 6/10, 1914.

HISTORY OF MOTHER

Born February 26, 1914.
Normal feeding.

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[1] *t* = treated. *n* = normal.

[2] 1/26–2/16 means: ♂ and ♀ were kept together from January 26 until February 16.

Final mating which gave living offspring

♂*t* × ♀*n* 6/12–7/6, 1914. 4 young born July 10, 1914. 2 young died after 3 days; 2 young living, undersized (figs. 4, 5).

SUMMARY OF CASE II

The father immediately after the thyroid treatment had ceased, was bred to the non-treated mother. 4 young were born 30 days after the last thyroid dose had been given. 2 young died soon, 2 undersized ones are living.

In this case the father only received thyroid *before* mating.

III. ♀*t* × ♂*n*.

HISTORY OF FATHER

Born December, 1913.
Normal feeding.

HISTORY OF MOTHER

Bought November, 1913, about 3 months old.
Thyroid feeding started 1/6, 1914.

1. Mating ♀*t* × ♂*t* 3/21–5/15, 1914; no pregnancy.
Thyroid feeding stopped 5/15, 1914.

Final mating which gave living offspring

♀*t* × ♂*n* 5/15–9/6, 1914. 6 young born 9/6, 1914. 2 young die 10/13, 1914. 4 living, small size (figs. 6, 7, 8; control figure 9). At the age of 94 days they weighed 46, 50, 58, 83 g resp. Normal rats of this age weigh from 120–150 g.

SUMMARY OF CASE III

(a) While under treatment the mother was bred unsuccessfully to a treated ♂.

(b) The treated mother immediately after the thyroid treatment ceased was bred to the non-treated father. 6 young were born 113 days after the last thyroid dose had been given. 4 young, smaller than the normal, are living.

In this case the mother only received thyroid *before* mating. She required three months to recover from the thyroid influence.

IV. ♂*t* × ♀*n*.

HISTORY OF FATHER

Born July 24, 1913.
Thyroid feeding started 3/21, 1914.

1. Mating ♂*t* × ♀*t* 3/21–5/15, 1914; no pregnancy.
Thyroid feeding stopped 5/15, 1914.

HISTORY OF MOTHER

Born May 26, 1914.
Normal feeding.

1. and 2. Matings: Bred to two normal males successively. 6 young and 8 young.

Final mating which gave living offspring

♂*t* × ♀*n* 5/15–9/10, 1914. 3 young born September 10, 1914. Undersized. At the age of 90 days they weighed: 52, 63, 99 g resp. Normal rats at this age weigh from 120–150 g.

SUMMARY OF CASE IV

(a) While under treatment the father was bred unsuccessfully to a treated ♀.

(b) The normal mother was bred successfully to 2 normal ♂.

(c) The treated father immediately after the thyroid treatment had ceased was bred to the normal mother.

3 young were born 117 days after the last thyroid dose was given.

In this case the father only received thyroid *before* mating. He required three months to recover from the thyroid influence.

To this list must be added a number of cases in which young were born alive, but died after puberty.

V. ♂*t* × ♀*n*.

HISTORY OF FATHER

Bought January, 1913. About 3 months old.
Thyroid feeding started January 13, 1913.

1. Mating: ♂*t* × ♀*t* 4/7–4/28, 1913; no pregnancy.

HISTORY OF MOTHER

Bought January, 1913. About 3 months old.
Normal feeding.

Final mating which gave offspring

♂*t* × ♀*n* 4/7–4/28, 1913. 5 young born 5/1, 1913.

HISTORY OF THE YOUNG

Born May 1, 1913.

1. Young died after 9 days. Very frail.

4 other young started on thyroid diet October 30, 1913. They were all undersized and rather feeble. One dragged the hind legs. When 84 days old they weighed: 57 g, 58 g, 60 g, 66 g, resp. Normal rats at this age weigh from 110–140 g.

2. Young died November 10, 1913.
3. Young died December 3, 1913.
4. Young died December 5, 1913.
5. Young died December 21, 1913.

SUMMARY OF CASE V

(a) While under treatment the father was bred successfully to a treated ♀.

(b) While under treatment the father was bred to the non-treated mother.

5 young were born 24 days after mating. 1 died 9 days old, 4 frail and undersized lived several months.

In this case the father only received thyroid up to the time of mating.

VI. ♂t × ♀t.

HISTORY OF FATHER

Bought January, 1913. About 3 months old.
Thyroid feeding started January 13, 1913.
1. Mating: ♂t × ♀t_3 4/7–4/28, 1913; no pregnancy.
2. Mating: ♂t × ♀n 4/7–4/28, 1913. 5 young 5/1, 1913 (case V).

Final mating which gave offspring

♂t × ♀t_3 5/26–9/14, 1913.
(Female in this successful mating is the same as in the first unsuccessful mating).
Thyroid feeding stopped June 26, 1913.

HISTORY OF MOTHER

Bought January, 1913. About 3 months old.
Thyroid feeding started January 13, 1913.
1. Mating: ♀t × ♂t_2 4/7–4/28, 1913; no pregnancy.

Final mating which gave offspring

♂t × ♂t_2 5/26–9/14, 1913.
Thyroid feeding stopped 6/26, 1913.

HISTORY OF THE YOUNG

7 young born September 14, 1913. Very frail and undersized. These young were started on thyroid October 31, 1913.

2 die November 10, 1913.
3 die November 12, 1913.
2 die November 15, 1913.

SUMMARY OF CASE VI

(a) While under treatment the father was bred unsuccessfully to the treated mother.

(b) While under treatment the father was bred to a non-treated ♀ (see case V).

(c) The treated father and treated mother (under a) were again mated. The thyroid treatment ceased 31 days after mating.

7 young were born 81 days after the last thyroid dose was given.

The treated parents required two months to recover from the thyroid influence.

VII. ♂t × ♀n.

HISTORY OF FATHER

Born July 21, 1913.
Thyroid feeding started January 6, 1914.

1. Mating: ♂*t* × ♀*t* 1/26–2/16, 1914; no pregnancy.
2. Mating: ♂*t* × ♀*t* 2/19–3/10, 1914; no pregnancy.
3. Mating: ♂*t* × ♀*t* 3/21–5/15, 1914; no pregnancy.

Thyroid feeding stopped May 15, 1914.

Final mating which gave offspring

♂*t* × ♀*n* 5/15–6/9, 1914. 10 young born 6/10, 1914. All die during June.

SUMMARY OF CASE VII

(a) While under treatment the father was bred unsuccessfully to 3 treated ♀.

(b) The treated father, the thyroid treatment of which ceased on the mating day, was mated to the non-treated mother.

10 young were born 26 days after the last thyroid dose was given. All died within 2 weeks.

In this case the father only received thyroid *before* mating.

VIII. ♀*t* × ♂*n.*

HISTORY OF FATHER

Born May 26, 1913.

1 and 2. Matings: ♂ bred to 2 normal females successfully. 8 and 7 young.

HISTORY OF MOTHER

Born July 21, 1913.

1. Mating: Bred to a normal male. 6 young born 1/26, 1914. Thyroid feeding started March 21, 1914.
2. Mating: ♀*t* × ♂*t* 3/21–5/15, 1914; no pregnancy.
   Thyroid feeding stopped May 15, 1914.

Final mating which gave offspring

♀*t* × ♂*n* 5/15–10/14, 1914.
5 young born 10/14, 1914, all eaten up by 10/19, 1914.

SUMMARY OF CASE VIII

(a) The non-treated father was bred successfully to 2 non-treated ♀.

(b) Before thyroid treatment began the mother too was bred successfully to a non-treated ♂.

(c) While under treatment the mother was bred unsuccessfully to a treated ♂.

(d) Finally the treated mother, immediately after the thyroid treatment had ceased, was bred to the non-treated father.

5 young were born 151 days after the last thyroid dose was given.

In this case the mother only received thyroid *before* mating. She required over four months to recover from the thyroid influence.

These eight cases reported here as well as numerous others of which the detailed history cannot be given leave no doubt that the excess of thyroid in the system greatly interferes with the breeding qualities of the animals. Twenty-four matings in which both the parents were treated resulted in failure, 2 in which the male alone had been treated and 4 in which the female alone received thyroid food, all in all 30 matings. Yet out of these 14 males, 7 had been tested and given offspring previously to the treatment, and out of the 16 females 9 had been tested and found fertile.

In a number of cases the female died before pregnancy began or before a live litter was born. The length of time these females were kept with the males is given in the following table:

1.	♀ dies after 2 days		Thyroid given after mating.
2.	♀ dies after 2 days		
3.	♀ dies after 7 days		
4.	♀ dies after 19 days	no pregnancy	
5.	♀ dies after 21 days		
6.	♀ dies after 21 days		
7.	♀ dies after 38 days		
8.	♀ dies after 57 days 6 fetuses		
9.	♀ dies after 67 days 7 fetuses		No thyroid given after mating.
10.	♀ dies after 107 days pregnant		
11.	♀ dies after 112 days pregnant		

The continuation of this table gives the time that elapsed between mating and birth of live litter:

12.	Young born after 24 days ♀ *normal* All die early	No thyroid given after mating.
13.	Young born after 26 days ♀ *normal* All die within two weeks	
14.	Young born after 27 days ♂ fed on normal food full month previous to mating ♀ fed on thyroid 2 months only	
15.	Young born after 28 days ♀ *normal* 2 die within a week	
16.	Young born after 110 days 87 days after thyroid treatment	Thyroid feeding continued for 33 days after mating.

17. Young born after 113 days
    ♂ normal
18. Young born after 117 days
    ♀ normal
19. Young born after 151 days
    ♂ normal

No thyroid given after mating.

This enumeration of 19 matings shows that pregnancy did not set in until several weeks after the discontinuation of the thyroid feeding. Whether or not copulation without fertilization took place before that time, cannot be stated. Surely attempts to copulate were made by the several males.

In those cases in which the litter was born within less than 30 days after mating,[3] the mother was normal, viz., not fed on thyroid (Nos. 12, 13, 15). Still the young died soon after birth. In the one case in which both parents had been treated and yet pregnancy set in early (No. 14), the male had been fed on normal food one month previous to the mating, while the female had been treated with thyroid for two months only. In cases 17–19 one of the two parents was normal, but it took the treated partner over three months to recover from the thyroid influence.

Thus under no circumstances will the continuation of the thyroid treatment allow pregnancy to set in. Numerous matings of this kind resulted in failure. Also in case 16 fertilization did not occur until 63–65 days after the thyroid feeding was stopped.

The feeding to rats of fresh thyroid tissue shows its effect in three different ways:

1. When the dose is too large, all the well known symptoms of hyperthyroidization become evident, viz., emaciation, diarrhoea, muscular weakness and finally cachexia leading to death. The hair becomes yellowish, stands erect, sometimes falls out in patches, in short the entire coat looks ragged.

2. When the dose is so regulated as to keep the animals in apparently good health—the fur will always become shabby then the animals do not breed. Not one mating of both parents treated gave any result, when the feeding continued after the animals had been placed together. Pregnancy was always

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[3] The gestation period of the rat is from 21–23 days.

delayed, since fertilization did not occur until several weeks after the application of the thyroid had been discontinued.

3. Did pregnancy finally occur, it resulted

(a) In abortus,

(b) The young died soon after birth,

(c) In very late pregnancies, the young show a diminished tendency to grow. Although they are not especially frail, they keep in relative size behind the normally fed rats of the corresponding age.[4]

The question now presents itself, whether the symptoms enumerated under (2) and (3) are simply the consequences of the general weakening effect of the thyroid food (1), or whether the genital glands have been affected so as to injure the sex cells. From the various reports in the literature on the interaction of thyroid and genital glands, especially under pathological conditions, this question is justified.

It may at once be said that the delay of pregnancy (2) is in all probability due to the weakening effect of hyperthyroidization. It seems, however, that this cannot fully account for the effects of the thyroid treatment, as stated under (3). It is reasonable to hold the latter responsible for the death of the several mothers during pregnancy and the reduced vitality of the offspring of early pregnancies. But the slackened growth of the young of late pregnancies can hardly be solely due to the weak condition of the mother. It can surely not be due to that condition in those matings in which the father only had been treated, while the mother was a normal female. It seems rather, although at present this is a mere assumption, as if the tendency to grow, inherited from the two parents, had been checked in some way. Former experiments already reported and some under way clearly show that the rate of growth and differentiation of a generation can be influenced by the application of thyroid, but there is no evidence yet that this influence can be transmitted to the second generation. If it could be shown that the rats of the second generation, although stagnant in growth, differentiate and mature at the same rate as or faster than normal rats, the proof for the correctness of the above assumption could be given.

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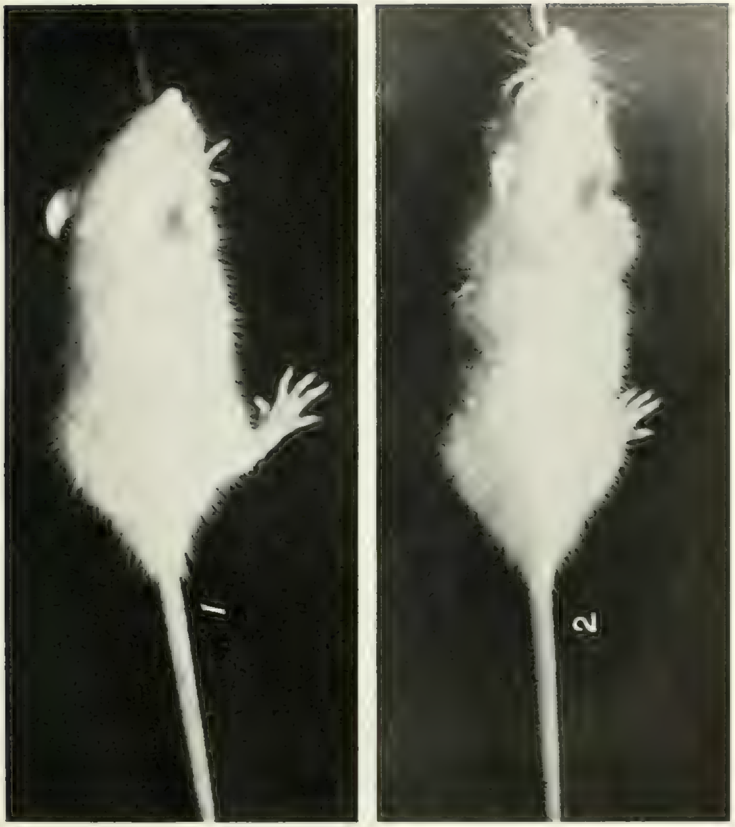
[4] There is considerable variation in size and weight between the several stocks of white rats raised in different laboratories. For information on this point I am much obliged to Professor H. H. Donaldson, of the Wistar Institute. I also refer to his various papers on this question. The thyroid treated rats do not in a given time reach the length and weight of our normal rats. Compare illustrations.

This preliminary report is supposed to state only some of the actual observations so far made. The histological data concerned will be reported, when the experiments will have been carried sufficiently far.



Figs. 1 and 2. One-third reduction. 2 rats 97 days old. Father and mother treated with thyroid.

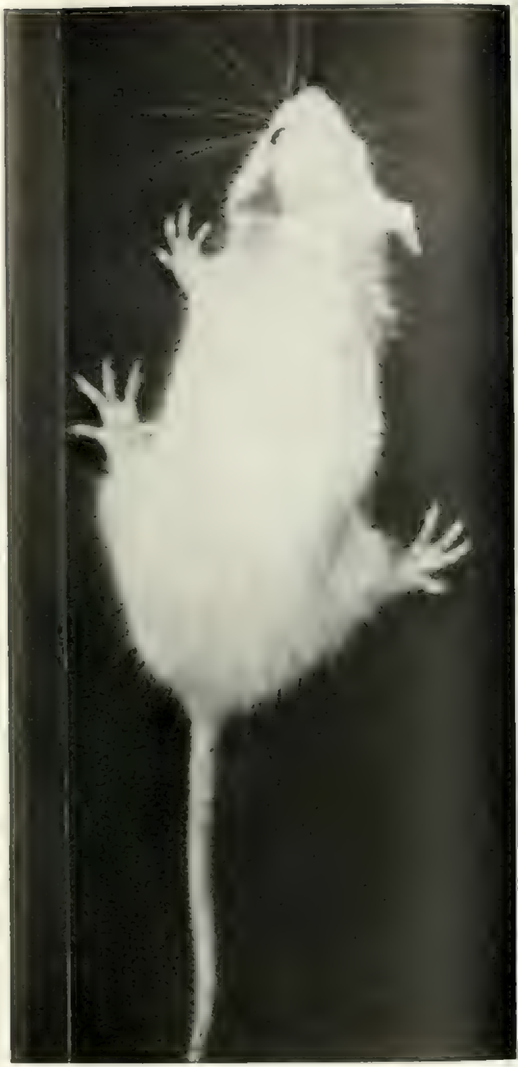


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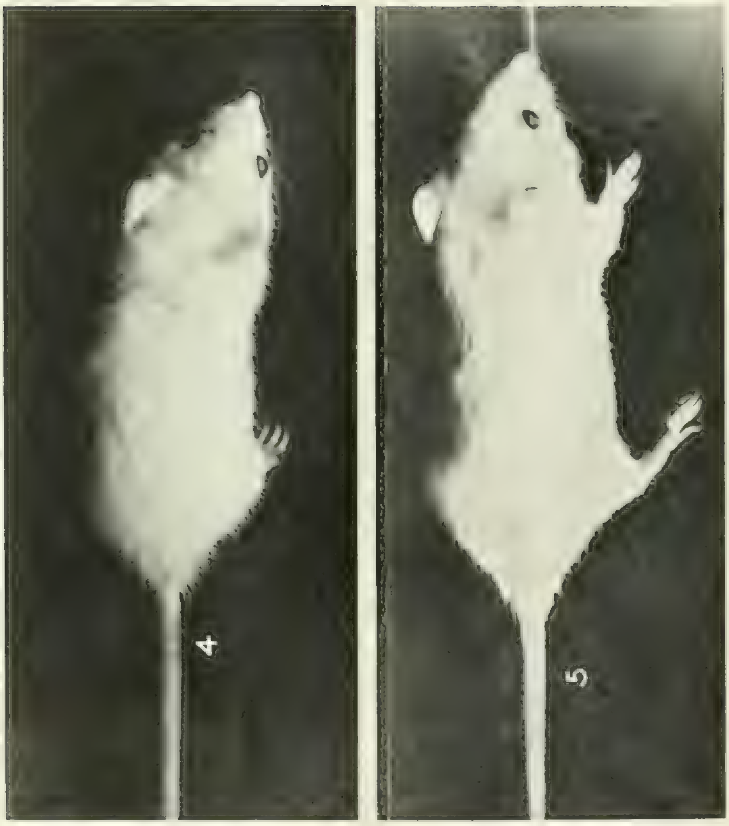
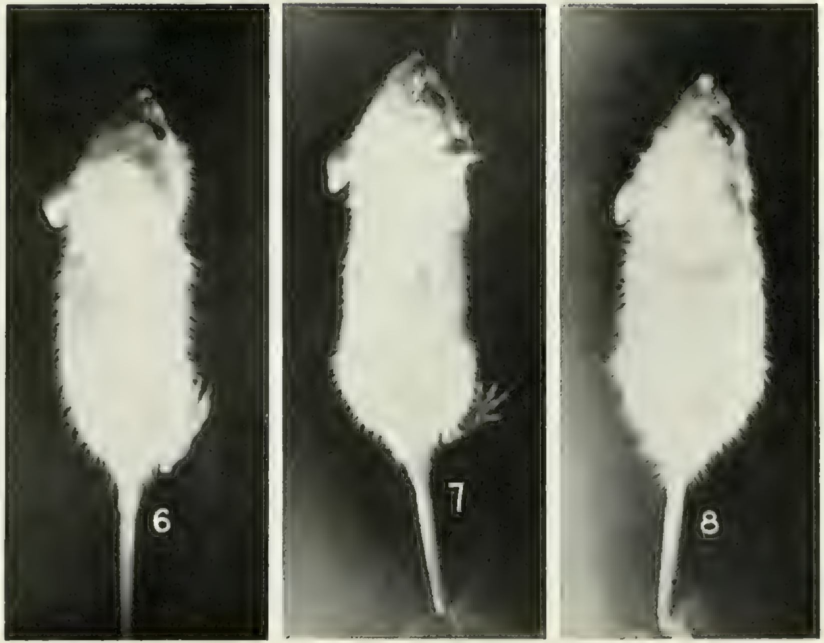
Fig. 3. One-third reduction. Normal rat 91 days old.



FIGS. 4 and 5. One-third reduction. 2 rats 96 days old. Father treated with thyroid.



FIGS. 6-8. One-third reduction. 3 rats 38 days old. Mother treated with thyroid.

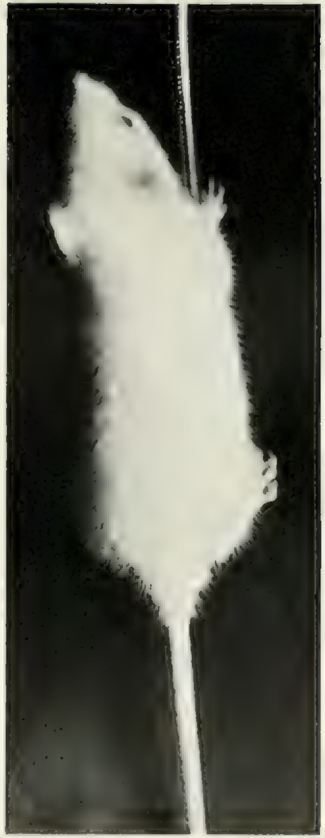


FIG. 9. One-third reduction. Normal rat, 34 days old.

Reprinted from The Anatomical Record, Vol. 9, No. 1, 1915

21. Feeding experiments on rats. J. F. Gudernatsch, Department of Anatomy, Cornell University Medical College, New York City.

Based upon the results obtained by feeding the internally secreting glands to amphibians, these experiments are being continued, this time with mammals. The glands are given to white rats in stated portions, at regular intervals. A preliminary account of some observations on the thyroid-treated animals may here be given.

Beef thyroid was fed in portions small enough to keep the animals in fairly good health; 1 gram a week was given, in some experiments 1 gram in 5 days. The application of even so small doses of thyroid sometimes produced slight symptoms of hyperthyroidism; however, the animals kept well enough to have offspring.

The following enumeration gives the records of 8 successful matings; in the first 4 cases the offspring are still living, while in the remaining 4 the young died, at stated dates.

Case I: ♂ *t* × ♀ *t*.[1] (a) While under treatment the father was bred to 3 treated ♀; no result. (b) After discontinuation of the thyroid treatment the father was bred to a non-treated ♀: 10 young were born after 26 days, all so frail that they died within a week. (c) One month after thyroid treatment of the father and immediately after thyroid treatment of the mother the two were mated; 3 young were born 54 days after the father and 29 days after the mother had received their last dose of thyroid. The young are much smaller than the normal rats of equal age.

Case II. ♂ *t* × ♀ *n*. After discontinuation of the thyroid treatment the father was bred to the non-treated mother; 4 young were born 30 days later; 2 died very soon, 2 undersized ones are living.

Case III: ♀ *t* × ♂ *n*. (a) While under treatment the mother was bred to a treated ♂; no result. (b) Immediately after thyroid treatment the mother was bred to the non-treated father; 6 young were born 113 days later; 4 undersized ones are living. The mother required 3 months to recover from the thyroid influence.

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[1] *t* = treated; *n* = normal.

Case IV: ♂ *t* × ♀ *n.* (a) While under treatment the father was bred to a treated ♀; no result. (b) The normal mother was bred to 2 normal ♂; 2 litters. (c) After discontinuation of thyroid treatment the father was bred to the normal mother; 3 young were born 117 days later; they are undersized. The father required 3 months to recover from the thyroid influence.

Case V: ♂ *t* × ♀ *n.* (a) While under treatment the father was bred to a non-treated ♀; no result. (b) While under treatment the father was bred to the non-treated mother; 5 young were born 24 days later; 1 died 9 days old, 4 very frail and undersized lived about 7 months. When 3 months old they weighed 57, 58, 60 and 66 grams respectively; (65 to 70 grams is the average weight of a rat about 60 days old).

Case VI: ♂ *t* × ♀ *t.* (a) While under treatment the father was bred to the treated mother; no result. (b) While under treatment the father was bred to a non-treated ♀: 5 young (see Case V). (c) The treated father and treated mother (under a) were again mated; thyroid treatment ceased 31 days later; 7 young were born 81 days later; very frail and undersized; lived 2 months. The parents required 2 months to recover from the thyroid influence.

Case VII: ♂ *t* × ♀ *n.* (a) While under treatment the father was bred to 3 treated ♀; no result. (b) After discontinuation of the thyroid treatment the father was mated to the non-treated mother. Ten young were born 26 days later; all died within 2 weeks.

Case VIII: ♀ *t* × ♀ *n.* (a) The non-treated father was bred to 2 non-treated ♀; 2 litters. (b) Before thyroid treatment the mother was bred to a non-treated ♂; 1 litter. (c) While under treatment the mother was bred to a treated ♂; no result. (d) After discontinuation of the thyroid treatment the mother was bred to the non-treated father; 5 young were born 151 days later; all died within 5 days. The mother required 4 months to recover from the thyroid influence.

The history of these cases shows that the feeding of thyroid to rats greatly interferes with their breeding qualities. Twenty-four matings, in which both parents were treated, resulted in failure, 2 in which the female alone had been treated and 4 in which the female alone received thyroid food, in all 30 matings. Yet out of these 14 males 7 had been tested and given offspring previously to the treatment, and out of the 16 females 9 had been tested and were found fertile.

Table 1 gives the enumeration of several matings, which will show that pregnancy did not set in until several weeks after the discontinuation of the thyroid treatment, except when the female was non-treated. The number of days is given that elapsed between the placing together of the parents and the birth of a live litter (or death of the female). The gestation period of the rat is from 21 to 24 days.

Thus under no circumstances will pregnancy set in during the thyroid treatment (cases 3 to 7, 15); after discontinuation of the thyroid treatment, the animals usually required several weeks to recover from the thyroid influence (cases 11 to 18).

TABLE 1

(1) ♀ dies after 2 days		Thyroid given after mating
(2) ♀ dies after 2 days		
(3) ♀ dies after 7 days	No pregnancy	
(4) ♀ dies after 19 days		
(5) ♀ dies after 21 days		
(6) ♀ dies after 21 days		
(7) ♀ dies after 38 days		
(8) ♀ normal; young born after 24 days; all die early		No thyroid given after mating
(9) ♀ normal; young born after 26 days; all die within two weeks		
(10) ♀ normal; young born after 28 days; 2 die within a week		
(11) ♀ dies after 57 days; 6 fetuses		
(12) ♀ dies after 67 days; 7 fetuses		
(13) ♀ dies after 107 days; pregnant		
(14) ♀ dies after 112 days; pregnant		
(15) young born after 110 days, 87 days after thyroid treatment		Thyroid feeding continued for 33 days after mating.
(16) ♂ normal; young born after 113 days		No thyroid given after mating
(17) ♀ normal; young born after 117 days		
(18) ♂ normal; young born after 151 days		

Only one mating of both parents previously treated gave offspring after 29 days. However, the treatment of the father had ceased one month before the mating time.

The feeding to rats of fresh thyroid tissue shows its effect in three different ways:

1. When the dose is too large, all the well-known symptoms of hyperthyroidization become evident, viz.: emaciation, diarrhoea, muscular weakness and finally cachexia leading to death. The hair becomes yellowish, stands erect, sometimes falls out in patches, in short the entire coat looks ragged.

2. When the dose is so regulated, as to keep the animals in approximately good health—the fur will always become shabby—then the animals do not breed. *Not one mating of both parents treated*, after the animals had been placed together, *gave any result*. Pregnancy was always delayed, since fertilization did not occur until several weeks after the application of the thyroid had been discontinued.

3. Did pregnancy finally occur, it resulted (a) in abortus; (b) the young died soon after birth; (c) in very late pregnancies, the young show a diminished tendency to grow. Although they are not especially frail, they keep in relative size behind the young of normally fed rats.

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THE ACTION OF ULTRA-VIOLET RAYS UPON THE FROG'S EGG

I. THE ARTIFICIAL PRODUCTION OF SPINA BIFIDA

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SIXTEEN FIGURES

While the method of the experimental embryological investigation detailed in the succeeding pages is of importance because of the constancy with which the condition of spina bifida may be produced in the embryo, the real value of the method and of its results lies in the interpretation which is thereby rendered possible of the physiological value of the several component portions of the fertilized ovum. The really fundamental problems to be determined by investigations of this nature are, first, whether the fertilized ovum is to be regarded as a composite structure made up of various system- or organ-anlagen, or the chemical progenitors or 'ferments' of such, distributed in definite and, perhaps, constant positions throughout the cytoplasm. Or, second, is the ovum to be considered in the sense of a unicellular organism, differing in no great respect from the physiological structure of unicellular organisms in general but possessing that specific potential to elaborate 'ferments' and pro-anlagen at successive genetic stages, and, ultimately the anlagen of the later developmental stages? In the former instance it is presumed that the embryonic parts are pre-localized in the cytoplasm of the ovum and make their appearance, in the words of Lankester, as "a sequel of a differentiation already established and not visible." In the second assumption the embryonic parts are unrepresented in the ovum, the regions of the cytoplasm being then, so far as the future embryo is concerned, of equipotential value. It appeared to the author that a means by which a limited area of cytoplasm could be destroyed and yet left in its original relations to surrounding parts would afford a solution to this question. Accordingly, recourse was made to ultra-violet rays of such a degree of intensity as to cause the disorganization of the cytoplasm in from one to thirty seconds and of such a degree of concentration as to influence limited surface areas. Acting upon the suggestions made by Prof. E. H. Merritt, an apparatus was constructed which met these requirements fully.[1]

This apparatus consisted of a large induction coil actuated by a 110-volt direct current reduced by an unknown resistance. The potential, moreover, was raised by means of several Leyden jars shunted between the electrode wires. The terminals were made of iron, and were spaced about 5.0 mm. The eggs used for the purpose were those of the various forms of frogs occurring in the neighborhood of Ithaca, New York. These were obtained early in the morning, as soon after laying as was possible. At the time at which they were influenced they were in the undivided stage. Development was allowed to progress in the laboratory in some instances, and the eggs influenced at several later developmental stages, but no egg further along in its cycle than about the 64-cell stage was used. Furthermore, care was taken to reject such eggs as were collected late in the laying season for that particular species of frog, and particularly those located near the center of the egg bunches, specifically to avoid dealing with those possessing a tendency towards abnormal development. In preparation for exposure to the rays, the eggs were freed from their jelly, which had been found impervious to the light, and placed under a perforated tinfoil diaphragm. The perforations differed in size in different experiments. After the egg had been rotated so that a predetermined part had been brought directly under the center of a circular perforation in the diaphragm, both were then brought under the electrodes of the apparatus and the circuit closed.

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[1] At this point I desire to express my indebtedness to Professor Merritt for helpful suggestions and to the Department of Physics of the University for the use of the apparatus with which the experiments mentioned in this paper were conducted.

While in the numerous experiments conducted, the various portions of the white and of the black hemisphere and of the equator of the frog's eggs were influenced in order, the author decided to limit the scope of this present communication to those effects produced by the rays when influencing the white hemisphere and the equator of the egg. Indeed, in addition to the significance of the findings of the investigation in the interpretation of the larger problem of ovum structure, the immediate purpose in presenting this paper is to establish the fact that the condition of spina bifida may be produced at will by this method.

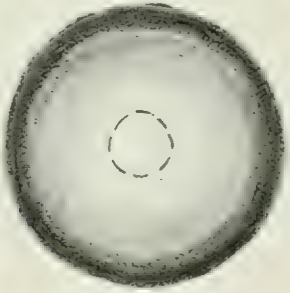


Figure 1

The aperture in the diaphragm used for this experiment was 0.4 mm. in diameter. The eggs averaged 1.7 mm. in diameter. Consequently but a small surface area proportionately of the total area of the egg was influenced. The latter amounted to 425.0 sq. mm. whereas but about 5.0 sq. mm. of this surface could be influenced by the rays. The relative sizes of these areas is brought out more clearly by reference to figure 1, which represents by a broken-line circle the portion of the surface area of the egg sphere illuminated. Further, it was found that the depth of penetration of the 0.4 mm. pencil of rays depended upon the length of exposure to the light. Uniform exposures of 30 seconds were employed in this series. A section through an egg so influenced is shown in figure 2, in which the depth to which the rays had penetrated is represented by the shaded portion on the right of the sketch. In this instance the rays passed in the plane of the section and at right angles to a tangent at the center of the surface of the affected area. The direct results of the illumination were corroborative of those previously observed by other investigators using violet rays, such as granulation of the chromatin and certain degenerative changes noted in the cytoplasm. No attempt was made, however, to study this aspect of the influence of the rays. It was noted in extremely long exposures of from 1 to 10 minutes, that masses of protoplasm were in some instances extruded upon the egg surface, retaining, however, a slender connection with the main mass of the egg. In instances of such exovation, the egg died early after having made but little developmental progress. Such an exovate is shown in figure 3. It is of importance to note the fact demonstrated by the sketch that the mass of the exovate was approximately equal to that of the influenced area of the egg (compare with figure 2). The most plausible inference to be drawn from this phenomenon, in the terms of the interpretation of the ovum as an organism, seems to be that of an effort on the part of the ovum to rid itself of the chemically altered or dead protoplasm which can only act as a hindrance to its further developmental progress.

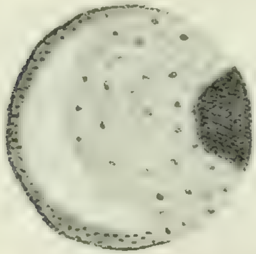


Figure 2

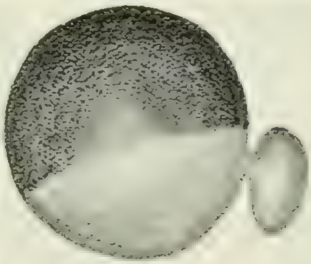


Figure 3

Reference to various series of experiments selected at random bring out the value of the method in the constancy of production of the condition of spina bifida. In one series of thirty-one 16-cell eggs used at the beginning of the experiments and exposed to the 0.4 mm. ray for 30 seconds each—various regions of the equator and of the white hemisphere being influenced—twenty-one developed abnormally, and but ten normally. Of the abnormal embryos, eight presented the condition of spina bifida. In another and later series of fifteen eggs in the 4-cell stage, influenced in the same manner, none developed normally. Most of these died during the early stages. Four, however, lived to swimming forms with two tails. Later in the spring, after the technique had been still further perfected and the eggs of the green frog were available, from which it was possible to remove the enveloping jelly more readily and more completely, the percentage of spina bifida embryos rose. In one set of five undivided eggs influenced in a similar manner, one died about twelve hours after the experiment, having made no developmental progress, and the four others grew to swimming forms presenting the condition of spina bifida, each having two tails. This last instance is merely representative of the high percentage of these forms of malformation obtained when the white hemisphere is influenced by the ultra-violet rays.

As has been mentioned above, the most effectual barrier to the penetration of the rays was the investing jelly. When this was completely removed an exposure of 10 seconds was sufficient to influence the egg. The presence of a very thin layer, however, completely blocked the passage of the rays even during an exposure of as much as 10 minutes. The author attributes most, if not all, of the irregularities in percentage production of spina bifida embryos to the presence of this jelly. The later results of the experiments were sufficiently assuring to warrant the conclusion that, when it could be positively known that the rays under the above conditions had actually penetrated the ovum in the regions above mentioned, the condition of spina bifida could be invariably brought about in the developing embryo. Taking these difficulties into consideration, however, the percentage production of the condition ranged between 85 and 90 per cent of the total number of eggs used.

An observation that recurred repeatedly was to the effect that the developmental period required by the eggs was lengthened as a result of the rays' influence. Under laboratory conditions ordinarily from 3 to 4 days were sufficient for the appearance of the free-swimming tadpole-forms of the green frog; in the case of the experimented eggs, however, 5 or 6 days were required and in some instances 8 days. Furthermore, it was noted that during the 12 hours immediately ensuing upon the experiment the eggs seemed to have entered into a condition of temporary suspension of development, later resuming that process but with greatly lengthened tempo.

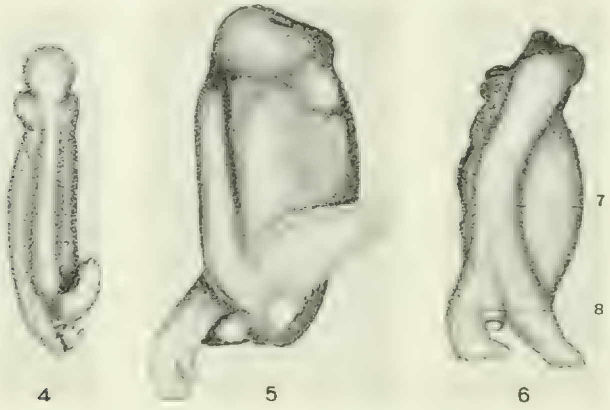


Fig. 4 A specimen of cauda bifida demonstrating the asymmetry of the tails. In this egg the rays had struck a portion of yolk farthest removed from the equator. The tails are provided, as is shown, with peculiar toe-like processes on their free extremities.

Fig. 5 In this tadpole the right tail encountered the body axis at an acute angle directed anteriorly. Two yolk plugs are to be seen, and the same splitting of the extremity of the left tail as was noted in figure 4.

Fig. 6 The lines 7 and 8 on this cauda bifida tadpole indicate the level of the cross-sections shown in the respective figures. In figure 7 the notochord lies ventral to the well differentiated neural tube. In figure 8 the asymmetry of the halved neural tube is shown, more particularly on the right side. Ventral to this lies the notochord, all traces of which are absent from the left half of the sketch. This right neural tube-half lay in the more actively used tail.

The further observation was made that free-swimming forms, such as are represented in figures 4, 5, and 6, seemed to be able to move about by the use of either tail, but that the swimming movements were more vigorous in one than in the other. This is an interesting fact in connection with the results obtained by study of the microscopical sections of the same specimens. In these it was learned that in the more favored of the two tails the neural tube was greater in diameter and extended a longer distance towards the tip of the tail. In some of the specimens, as is to be seen in figure 8, the notochord was limited to one tail. Figures 7 and 8 are cross-sections of the tadpole represented by figure 6. The right was the more active of the two tails during life. In figure 7 the neural tube, cut just cephalic to its bifurcation, is seen to be well differentiated, with the notochord lying ventral and adjacent to it. The section of figure 8 was taken immediately caudal to the bifurcation and shows the notochord confined to one (the right) tail.

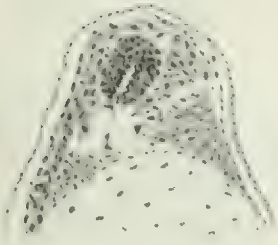


Figure 7

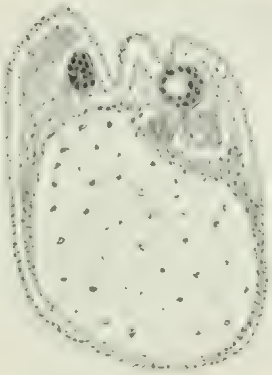
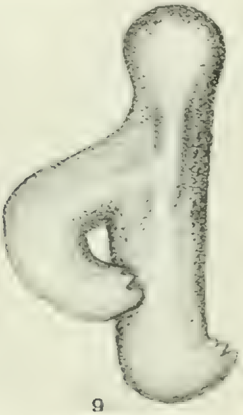


Figure 8

Several specimens presented a peculiar relation of one tail to the longitudinal axis of the body; such are figured in 5 and 9. In the latter figure the main axis of one tail joined that of the trunk at almost right angles, whereas the other tail coincided fairly well with the main body axis. In figure 5 the right tail met the body axis at an acute angle, looking forward. Such tails were, of course, useless from the functional standpoint; but their importance cannot be overestimated in furnishing exaggerated examples of the asymmetry of some of the types of spina bifida, such as were observed above in the cross-sections.

The study of the serial cross-sections demonstrated, furthermore, as these were followed in order caudally, that just posterior to the level of bifurcation of the neural tube each half tube destined for each tail presented an asymmetrical outline, the lateral wall being considerably thicker than the median. This is shown in part by the right neural tube in figure 8. As the series was followed farther caudally, however, a readjustment of the tube cells was observable, each half now becoming either a solid rod or a tube entirely symmetrical so far as the thickness of its walls was concerned; (see also figures 12 and 13).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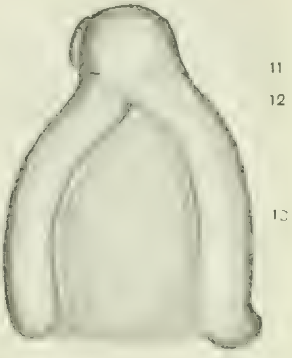

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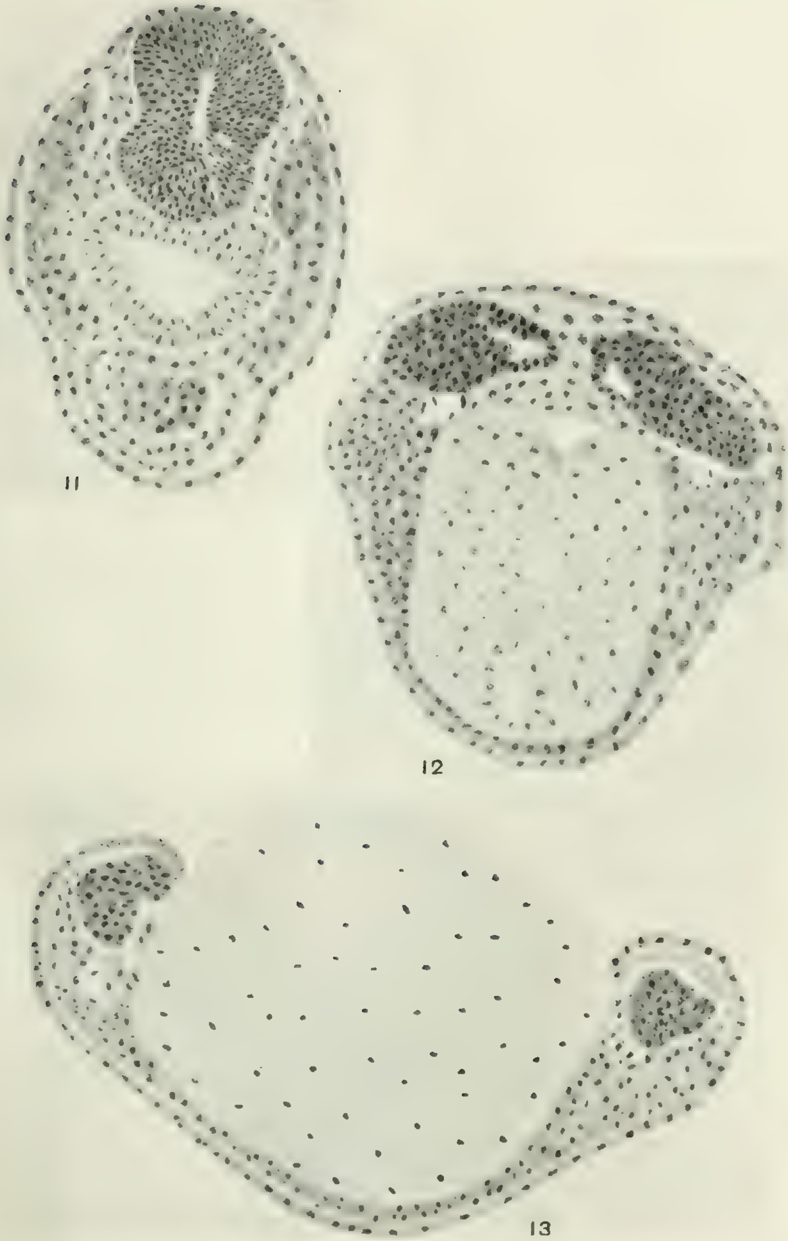
Fig. 9 This figure illustrates a marked instance of asymmetry with a division of the cord well anterior on the embryo. Here again the extremity of each tail is broken up into toe-like processes.

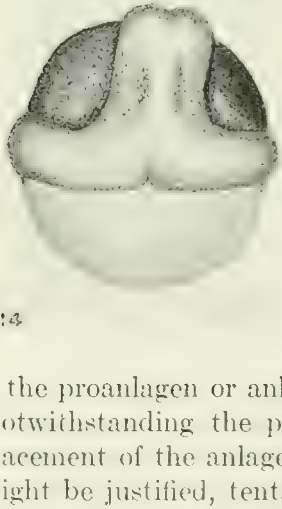
Fig. 10 In this embryo the rays had encountered an area well up on the equator in the median plane; hence, the bifurcation of the neural cord immediately posterior to the optic anlagen. The lines 11, 12 and 13 indicate the levels of the respective cross-sections illustrated by the succeeding figures.

The neural tube was caused to bifurcate at various levels, dependent upon the portion of the hemisphere influenced.



Figs. 11, 12, 13 In figure 12 the marked asymmetry of the neural tube halves immediately posterior to the level of bifurcation of the cord is shown. The neuroblasts become adjusted farther posteriorly, however (fig. 13) to form a more symmetrical tube or column of cells.

Where the rays struck close to or on the equator in the median plane of the egg the subsequent bifurcation was noted well forward towards the head region; figure 10 well demonstrates this fact. In figure 9 the point encountered was much lower down on the hemisphere, while in figures 6 and 4 it was still farther posterior. Such are really instances of cauda bifida. Another fact of the utmost significance was gained from studies of the histological sections. Since it was definitely ascertained that the rays had killed the portion of the egg illuminated, in no specimen was there to be observed, however, a deficiency or falling out of a portion of the neural tube, as one would expect if the proanlagen or anlagen had been encountered by the rays. Notwithstanding the possibility of post-generation, with a replacement of the anlagen thus rendered inactive, the conclusion might be justified, tentatively at least, that the proanlagen are not located in the early stages of division of the ovum either in the yolk hemisphere or along the equator, but are confined wholly to a region well up on the black hemisphere. Before referring in detail to the results of other investigations dealing with spina bifida it will be well to emphasize some general considerations which must be taken into account in connection with the production of abnormalities.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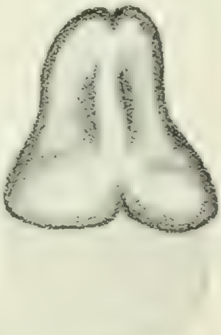
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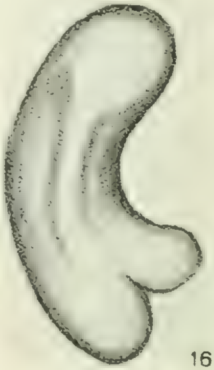
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It is taken for granted that there are many linking factors associating the developmental processes concerned in the production of spina bifida with those of other forms of malformation occurring in nature and produced experimentally. Accordingly, one cannot well study the one without taking into consideration those general fundamental physico-chemical factors of development underlying both, upon the disturbance of which the production of anomalous conditions is dependent. The fact is well supported that the processes of differentiation in the tadpole, as in some other forms, appears to be dependent upon a series of complex and progressive chemical reactions which are of the nature of oxidations. In the later stages of development we are dealing with an organism whose chemical constitution differs considerably if not completely from that of the undivided ovum. The results of such reactions and chemical changes become apparent in the differentiation of the various anlagen. Eggs placed in pure water free from chemical compounds which might enter into the chemical structure of the organism, but supplied with a liberal amount of oxygen, can and do undergo the several processes of differentiation, finally hatching and swimming about. From that time on, however, when growth of the differentiated parts is initiated the organism requires a supply of various chemical substances for its appropriation. In this connection, it is significant that the great majority of defects produced experimentally are referable to changes in the differentiative stages of the embryo and only secondarily to defective growth phenomena.

Bearing in mind, then, the chemical modifications occurring during the differentiating cycle of the embryo, it is fair to assume that at certain specific times in the genesis of the anlagen their chemical composition is such as to render them more ready participants in chemical reaction with the chemical agent employed. Results so obtained cannot be cited without reservation as applying to the state of the undivided ovum, consequently much of the chemical work conducted along this line is subject to considerable qualification and limited in the insight which it furnishes into the constitution, chemical or physiological, of the ovum. Before the full truth can be established on this point, it must be demonstrated that the toxic action of the chemicals employed was exerted upon the egg during the undivided state and not afterwards; or, in other words, upon the chemical 'ferments,' or proanlagen, and not upon the anlagen cells when they have attained what might be termed a condition of chemical completeness. Only by this means can we decide between the two conceptions of the ovum; as an organism elaborating its organ anlagen at succeeding developmental periods, or as a composite, mosaic-like structure.

While the artificial production of defects has been known for a long while to be possible, but very few have succeeded in advancing any plausible explanations covering the instance of spina bifida embryos. There are two aspects to the problem of the artificial production of spina bifida which are brought out in a review of the literature dealing with this subject. The first consideration is whether we are dealing with the action of an external agent upon some specific substance in the egg; and the second, whether the nature of this reaction is specific, referable to the agent alone, which possibly reacts upon the egg as a whole.

Morgan in 1894 and O. Hertwig in the year following were both successful in the production by chemical means of a large percentage of embryos showing the defect of spina bifida. That 0.625 per cent solutions of sodium chloride should produce so high as 50 per cent of this form of malformation was an argument in favor of a definite specific chemical or physical property of the compound. Prior to this date observers had recorded only occasional instances of this defect and had failed to give a convincing indication of the nature of the upset in the physicochemical factors concerned. Roux was the first to call attention to the occurrence of spina bifida among frog's eggs, owing, apparently, to conditions found in nature. Panum recorded 38 instances of spina bifida in chicks, among 404 monsters produced. He, with Dareste and Féré, obtained monsters of various kinds by the employment of variations in temperature (as did Hertwig), by varnishing the egg shells, by shifting the long axis of the egg to the vertical, by traumatic injuries, shaking, magnetism, electrical means, various gases, vapors of lavender and by injecting different toxines and chemicals, such as turpentine, aniseed, absinthe, and cloves into the white of the egg. Their inability to associate any given deformity* with a known and controllable cause led to a failure in the analysis of the normal developmental factors of the embryo. Richter found three instances of spina bifida among several hundred hen's eggs upon which he had experimented. Spemann, however, produced two-tailed embryos by simply tying a ligature between the two blastomeres, demonstrating the bilaterality of the anlagen but throwing no light on the nature or antero-posterior extent of the organ-building substances. Fol, Rauber, Born, and O. Hertwig attributed the duplicity to double fertilization. This explanation was too compromising regarding the anterior portion of the embryo, and later was found to be unnecessary. Godlewski's experiments with reduced pressure, and Herbst's with lithium salts, Morgan's with the centrifuge, Samossa's with atmospheres of nitrogen and of hydrogen, and Wilson's with Ringer's solution and with sodium chloride, furnish additional evidence of the diversity of ways by which this abnormal condition may be produced.

In this connection, it is interesting to note that Mall has reported 12 instances of spina bifida among 163 pathological human embryos, attributing as a possible cause of the condition, faulty implantation of the embryo. Analysed still further, however, by analogy to the conditions found among lower vertebrates, it seems possible that the human ovum, too, requires but little else than a good supply of oxygen for its differentiation during the early stages of development. At this time the causal forces are operative for the production of spina bifida. Undoubtedly, a deficiency in the supply of oxygen could be brought about by the imperfect imbedding of the ovum in the uterine mucosa. Bearing this fact in mind in connection with the features of differentiation of the ovum given on page 375, it seems superfluous to seek an explanation of the condition in man through the action of chemical substances or of altered temperature. Though the possibility of the direct or indirect dependence of the processes of oxidation upon the action of the latter agents must be admitted, reasoning from conditions as we find them in the frog, a sufficient and probably a more primal cause, at least, is referable to faulty oxidation.

Guthrie produced these defects by the use of strychnine, caffeine, and nicotine, as had Hertwig, but with concentration far below that of 0.625 sodium chloride. Jenkinson, however, tested out this question of the osmotic pressure of the salt solutions by employing a great variety of isotonic solutions of various salts, such as chlorides, bromides, iodides, nitrates, and sulphates of ammonium, lithium, sodium, potassium, calcium, barium, strontium, and magnesium, and, in addition, solutions of cane sugar, dextrose, urea, and gum arabic. He obtained spina bifida with especial success in his sodium chloride and sodium nitrate solutions. His conclusions are best given in his own words: "There is very little room for doubt, that the malformations in question may be due to some property of a salt other than its osmotic pressure." Bataillon had previously come to the conclusion that malformations were not specific to the means employed. Gurwitsch's belief was that halogens affected the position and development of the blastopore and of the brain, sodium chloride acting upon both, and sodium bromide upon the brain alone, whereas lithium chloride seemed selective on archenteron and blastopore.

It would appear, considering the production of this malformation by the diverse methods outlined above in connection with that detailed by this paper, that we were justified in concluding that in the question of specificity of reaction in the production of spina bifida the weight of argument at present refers the causative forces more particularly to an upset of a specific substance in the egg, rather than a specific action of the agent. It is in the conception of the changes which occur in the chemical composition of the ovum during its differentiation, as previously outlined, that we find support for this statement. It follows that the composition of any particular proanlage or anlage may be such at different stages of its chemical elaboration as to possess a marked affinity for widely varying chemical reagents. The developmental end-product of the reactions so brought about would be the same, e.g., spina bifida, notwithstanding the wide diversity of character of the chemical reagents employed.

Furthermore, since we cannot deny, we must take into account the possibility of a two-fold manner of production of spina bifida; the one, owing to an upset in the contents of the cells of the unpigmented hemisphere whose yolk is intended for the nutrition or elaboration of the other component, viz., the proanlagen or chemical ferments restricted to the cells of the pigmented hemisphere. For the other, we can assume the possibility of an interference in the function of these proanlagen as the result of the chemical reactions experimentally induced, apart from an upset referable to the composition of the nutritive yolk particles. The author's work, however, points out clearly that in the white hemisphere alone are resident sufficient causes for the production of the malformation, so that, while the possibility of an involvement of the proanlagen exists, the weight of experimental evidence points to the yolk hemisphere as the more vulnerable of the two. Jenkinson observed, for instance, that as the result of chemical action the yolk cells were primarily affected, and Godlewski, employing reduced pressure, came to the same conclusion. The disturbing influences of insufficient aeration and cold, as ascertained by Morgan and others, were noted first in the yolk cells, and to this same region Morgan attributes the causative factors in the results of the centrifuge, while Hertwig drew the same conclusions from studies of overripe eggs.

The production of an area of altered protoplasm, which serves as a mechanical check to the approximation of the lips of the blastopore during the backward progression of the latter, emphasizes very naturally the importance of synchronized tempo in the two processes directly concerned with the elaboration of the neural cord. Under normal conditions the differentiation of the neural anlagen (consequent upon the backward migration of the proanlagen) occurs apparently synchronously with the backward migration of the blastopore and fusion of its dorsal lips. These two processes are approximately coöperative in point of time, i.e., the anlagen of each half-tube become progressively differentiated in a backward direction at about the time when the half of the dorsal lip in which it is localized meets and fuses with its corresponding fellow of the opposite side. The two processes are not causally dependent upon each other, however, since differentiation takes place in the experimented eggs at about its former rate but now along the equator and not, as usual, parallel to the median plane.

The absence of the proanlagen and anlagen of the egg along the equator in the earliest stages of development of the ovum is sufficiently attested for by the ultra-violet method. Incidentally, it should be remarked that the restriction of these proanlagen at all times to the pigmented hemisphere seems to the author's mind a very significant fact. In this connection, it should be stated that ultimately the yolk mass is wholly drawn into the body of the embryo. Even though by this later process the neural tube halves may be approximated, subsequent fusion does not take place, however, since each half tube has postgenerated into a whole tube.

The conclusions reached by this method of experimentation upon the fertilized ovum, are, therefore: first, that the killing of a small localized area of the yolk hemisphere or of the region of the equator of the frog's egg produces invariably the condition of spina bifida in the embryo; and second, that the neural tube proanlagen, or formative substances, do not lie either in the yolk hemisphere or along the equator of the frog's egg, but are wholly restricted to the pigmented half of the egg. These proanlagen attain their definitive positions by a process of backward migration, the rate of which is synchronous with that of the backward progression of the dorsal lip of the blastopore. The action of the ultra-violet rays in destroying a small localized area of the yolk hemisphere or equator results from mechanical causes in an upset of the synchronism of the two factors, i.e., differentiation of the neural anlagen and approximation of the lips. The former proceeds at its normal tempo, while the latter is retarded. Consequently, the former, always restricted to the pigmented hemisphere, come to lie along the equator and are later carried towards the median plane by the subsequent approximation of the lips, but the half tubes, having already differentiated into whole tubes, do not subsequently fuse.

BIBLIOGRAPHY

BATAILLON 1901 Arch. Entw. Mech., Bd. 12.
BORN 1887 Bresl. ärztl. Zeitschr. für 1882, Bd. 14, Bd. 15.
DARESTE 1891 Recherches expérimentales sur la production artificielle des monstruosités, Paris.
FÉRÉ 1893–1901 Compt. Rend. Soc. Biol.
FOL 1879 Mém. de la Soc. de Phys. et d'hist. Nat., Genèvè.
GODLEWSKI 1901 Arch. Entw. Mech., Bd. 11.
GURWITSCH 1896 Arch. Entw. Mech., Bd. 3; Zeitschr. f. wissensch. Zool., Bd. 55, 1893.
HERBST 1897 Mitt. d. Zool. Station zu Neapel, 11, 1895; Arch. Entw. Mech., Bd. 5.
HERTWIG, O. 1896 Festschr. f. Karl Gegenbaur, Leipzig (Arch. f. mikr. Anat., Bd. 44, 1895).
JENKINSON 1906 Arch. Entw. Mech., Bd. 21.
KNOWER, H. McE. 1907 Anat. Rec., Amer. Jour. Anat., vol. 7.
LANKESTER 1877 Notes on embryology and classification.
LOEB 1893 Pflüger's Archiv., Bd. 54; Amer. Jour. Physiol., III, 1900.
MALL, F. P. 1908 Jour. of Morph., vol. 19.
MORGAN, T. H. 1894 Anat. Anz., Bd. 9, Quart Journ. Micr. Sci., vol. 35, no. 5
1897 The development of the frog's egg.
1909 Anat. Rec., vol. 3.
PANUM 1878 Untersuchungen über die Entstehung der Missbildungen, zunächst in den Eiern der Vögel, 1860, and Virchow's Arch., Bd. 52.
RAUBER 1878 Virchow's Archiv., Bd. 71, Bd. 73 and 74; Bd. 81, 1883.
RICHTER 1888 Verhand. der Anat. Gesellsch.
ROUX 1895 Gesammelte Abhandlungen, Leipzig.
SPEMANN 1903 Zool. Jahrb. Suppl.
WILSON, C. B. 1897 Arch. Entw. Mech. V.

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SEX DETERMINATION AND SEX CONTROL IN GUINEA-PIGS

THE observations, a short exposition of which is given here, were made on guinea-pigs, being used by Professor Stockard in heredity experiments. He very kindly placed the material at my disposal for this study, and I wish to express my appreciation of this favor.

These observations show that the sex of a guinea-pig is determined sometimes by two and sometimes by three factors, depending upon whether the mother has previously born young.

The first factor "A" is the sex tendency of the father. If the father has a male sex tendency, his sons will have a female tendency and his daughters a male tendency. If, on the contrary, the father possesses a female tendency, his sons will have male tendencies and his daughters female tendencies. In other words, sons exhibit the opposite and the daughters the same tendency as the father.

The second factor "B" is the sex tendency of the mother. A mother with a male tendency gives her daughters a female and her sons a male tendency. The mother with a female tendency gives her daughters a male and her sons a female tendency. Thus the transmission of the sex tendency from the female is also criss-cross in the same fashion as that of the male. The females inherit like tendencies from their father and the males like tendencies from their mother, whereas the males inherit the reverse tendency of their father and the females the reverse tendency of their mother.

The third factor "C" is confined to the female and is a change of sex tendency from litter to litter. This change in tendency manifests itself in the following way:

If the first litter contains only males, the mother acquires a female tendency for the next litter and vice-versa. This new tendency varies in strength, depending upon the number of young of one sex contained in a litter. The greater the number of males in a litter, the stronger the female tendency will be for the next litter. This tendency is still more emphasized if the mother is successively mated with males of a definite tendency, and therefore forced to produce more and more young of one sex.

The tendency of the various animals of a certain stock must first be ascertained in an experimental manner; given a number of undetermined males and females, each male must be mated with all the females and each female with all the males. After all the animals have been tested in this way, the results will show more males from some animals and more females from others. If, now, the offspring from these matings be grouped so as to take those animals which have come from more male producing fathers and their tendency be tested, it will be found that from the males more females will be produced and from the females more males. Provided the determination of the sex tendency for the first set of animals was absolutely correct, and if there was no other factor in action, the proportion of males to females should be as 75:25 from male producing males mated with females having different tendencies, and from female producing males the proportion is reversed. It is, however, very difficult to determine absolutely the sex tendency of an animal after only a few matings, and for this reason, some animals supposed to have a male tendency will probably have a weak female tendency, and *vice versa.*

In order to find with reasonable definiteness the tendency of animals, the tendency of the ancestors for three or more generations should be known. It must also be recalled, as explained above, that the third factor "C" in the female reduces the difference between the ratio numbers of her male and female descendants. As a result of this, the difference between the number of males and females considering only mother tendency is smaller than the difference between males and females in the light of only the father tendency. A male has a male tendency or a female tendency and always maintains it, whereas the female has a born male or female tendency, but in addition to this she has a second tendency to change her sex tendency from litter to litter. The number of males and females derived from the second generation was as follows:

I. Descendants of males whose fathers had a male tendency mated with mixed females; 39 males to 54 females, *i. e.,* 41.94 per cent. males to 58.06 per cent. females (sex ratio 72.20).

II. Descendants of males whose fathers had a female tendency mated with mixed females; 42 males to 23 females, *i. e.,* 64.61 per cent. males to 35.39 per cent. females (sex ratio 182.60).

III. Descendants of males whose mothers had a male tendency mated with mixed females; 64 males to 53 females, *i. e.,* 54.70 per cent. males to 45.30 per cent. females (sex ratio 120.83).

IV. Descendants of males whose mothers had a female tendency mated with mixed females; 12 males to 13 females, *i. e.,* 48 per cent. males to 52 per cent. females (sex ratio 92.30).

V. Descendants of females whose mothers had a male tendency mated with mixed males; 13 males to 18 females, *i. e.,* 41.94 per cent. males to 58.06 per cent. females (sex ratio 72.22).

VI. Descendants of females whose mothers had a female tendency mated with mixed males; 51 males to 43 females, *i. e.,* 54.25 per cent. males to 45.75 per cent. females (sex ratio 118.60).

VII. Descendants of females whose fathers had a male tendency mated with mixed males; 38 males to 36 females, *i. e.,* 51.38 per cent. males to 48.62 per cent. females (sex ratio 105.55).

VIII. Descendants of females whose fathers had a female tendency mated with mixed males; 33 males to 37 females, *i. e.,* 47.15 per cent. males to 52.85 per cent. females (sex ratio 89.18).

These figures show that in the sons the tendency received from the father is stronger than that coming from the mother, while in the daughters the opposite is true.

When one examines the descendants of animals whose fathers had a male tendency and mothers a female tendency, a higher difference in the relative number of males and females is found than from those cases in which the fathers alone had a male tendency. Twenty descendants of such male animals (father male tendency and mother female tendency) mated with mixed females consisted of four males and sixteen females, *i. e.,* 20 per cent. males and 80 per cent. females (sex ratio 25.00). From the females of the same type (father male tendency and mother female tendency) mated with mixed males, 29 males and 15 females were derived, *i. e.,* 65.90 per cent. males to 34.10 per cent. females (sex ratio 193.33).

From the second-generation males whose fathers had a female tendency and whose mothers show a male tendency when mated with mixed females were derived 32 males and 15 females, *i. e.,* 68.08 per cent. males to 31.92 per cent. females (sex ratio 213.33). From the females of the same type (father female tendency and mother male tendency) mated with mixed males were derived 6 males and 13 female descendants, *i. e.,* 31.58 per cent. males to 68.42 per cent. females (sex ratio 46.15).

Should one select males whose fathers had a female tendency and whose mothers had a male tendency and mate these with females whose fathers had a male tendency and whose mothers had a female tendency, a higher difference in the relative number of males and females will be found in their descendants than in any other possible case. From such combinations were derived nine animals, all males. In the same way, from the combination a male derived from a father, male tendency, and mother, female tendency, mated with a female from father, female tendency and mother male tendency, were derived six animals all of which were females.

This regulation in the inheritance of the sex tendency is especially interesting in affording an explanation of the manner in which the equilibrium is maintained between the number of male and female offspring of a given species. With each new generation each male animal has an opposite tendency from that of his father and each female animal an opposite tendency from that of her mother. It, therefore, follows that a disturbance of the equilibrium in one generation will tend to be restored by the opposite tendencies in the following generation. The above-mentioned change of sex tendency from litter to litter in the female leads to the same result. This third factor " C " regulates equilibrium from birth to birth so that any disturbance of a great degree is impossible.

The difference in the proportion between the sexes in different species may be due to the fact that in some species the father and mother have an equal influence on the determination of the sex of their offspring while in other species either the father or the mother may have the greater influence.

When father and mother have equal influence, the combination father, male tendency, with mother, female tendency, will give equal number males with females tendencies and females with male tendency, and the combination father, female tendency, with mother, male tendency, will give equal number males with male tendency and females with female tendency. In this way equal numbers of male and female descendants will be produced and equal numbers of the descendants will have male and female tendencies. In such a case the sex ratio should be 100 per cent. Should, on the other hand, the mother have the greater influence on the determination of sex, as seems to be the case in the guinea-pigs, then the number of descendants with a male tendency will be greater than the number of those having a female tendency, as the following scheme shows:

Father with male tendency mated with mother with female tendency will give more females (male tendency) than males (female tendency).

Father with a female tendency mated with a mother with a male tendency gives more males (male tendency) than females (female tendency). Therefore from either combination the greater number of offspring have a male tendency and as a result of this the sex ratio will be greater than 100.

In guinea-pigs it really seems that the influence of the mother is greater than that of the father, and this may be the explanation of the fact that the number of male guinea-pigs is greater than the number of females.

Finally, if the father has a greater influence on sex determination than the mother the number of descendants with a female tendency will be greater than the number with a male tendency, and consequently the sex ratio will be smaller than 100, as is shown by the following analysis:

Father with male tendency mated with mother with female tendency gives more males (female tendency) than females (male tendency). The father with female tendency mated with mother with male tendency gives more females (female tendency) than males (male tendency). Thus from either combination the greater number of offspring have a female-producing tendency and as a result of this the sex ratio will be less than 100.

Concerning the third factor, "C," the change of the sex tendency from litter to litter, a statistical examination shows the following results:

First: Relative number of male and female descendants after the birth of one or more females in one litter; 38 males to 12 females, *i. e.,* 76 per cent. males to 24 per cent. females (sex ratio 316.66).

Second: Relative number of male and female descendants after birth of one or more males; 13 males to 38 females, *i. e.,* 25.49 per cent. males to 74.51 per cent. females (sex ratio 34.21).

To determine further whether the sex-tendency of males showed any inclination to change from mating to mating the records were counted in the following manner:

Taking the matings of given males following matings that produced only female young, it was found that the product of such matings consisted of 22 males to 27 females, *i. e.,* 44.90 per cent. males to 55.10 per cent. females (sex ratio 81.48).

Taking the matings of given males following matings that produced only male young, it was found that the product of such matings consisted of 28 males to 24 females, *i. e.*, 53.84 per cent. males to 46.16 per cent. females (sex ratio 116.66).

This result is therefore the reverse of that shown by the females. Whereas the females show an opposite tendency following each litter, the males always maintain the same tendency.

Only those litters which were purely male or female were used in the above consideration. After a mixed litter of males and females, which is more common under natural conditions, there is not a pure, but also a mixed sex tendency. This fact renders the recognition of the "C" factor extremely difficult.

Such a characteristic change in tendency from birth to birth also seems to occur in other animals. The daphnids, for instance, seem to have some such regulation very definitely expressed.[1] In these organisms also the sex tendency changes from generation to generation as well as from birth to birth in such a way that not after each generation and each birth, but after a number of generations and births, differing with different species, the exclusive production of parthenogenetic female ceases and the first males appear. Doubtless we have in this an example of a change of the sex tendency, but its expression is quite different from that in the guinea-pigs.

From a theoretical standpoint, it is very important that coincidentally with the change of sex tendency in the summer eggs from female to male in *Moina rectirostris* var. *Lilljeborgii*, there is also a change in the color of these eggs from violet to blue.[2] This fact probably indicates that some chemical change occurs in the eggs at the same time that the change in the sex tendency takes place.

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[1] Papanicolaou, G., "Experimentelle Untersuchungen über die Fortpflanzungsverhältnisse der Daphniden," *Biol. Zentralbl.*, 30, 1910.

[2] *Ibid.*

At the present time I am endeavoring to complete my observations and to determine statistically the relative value of the three factors in different combinations. Since, however, the animals at my disposal are designed especially for the study of the degenerative influence of alcohol, it will, no doubt, require a long period of time to collect hundreds of selected cases, since so few animals of the generations later than the third are capable of reproduction.

This preliminary report is published with the hope that other investigators, having a large stock of different animals at their disposal, may further contribute to the solution of this problem in all its details.

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June, 1915

AN INSTANCE OF ACIDOPHILIC CHROMOSOMES AND CHROMATIN PARTICLES

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ONE PLATE (TWELVE FIGURES)

Studying the cytological literature, I have been unable to find a record of acid staining chromosomes in a normally dividing cell. In an investigation of sections of petromyzon larvae numerous mesenchyma and blood cells are seen which contain nuclei staining a uniform and brilliant red. These cells are scattered among other cells having nuclei of exactly the same structure, yet staining the usual deep blue color with the hemotoxilyn eosin stain. After studying these cells more closely, I found that the cells with the red nuclei were able to undergo mitotic division in the same manner as the cells with blue nuclei, the chromosomes in such cases staining red rather than the characteristic deep blue or black seen in the neighboring cells.

Such cells have been found as free blood cells in the blood vessels and also as mesenchyma cells in the pharyngeal and head regions of the larvae. Wherever found, aside from the peculiar property the nuclei show in the absorption of the acid dye, these cells are exactly similar to others in the region in which the nuclei stain characteristically with the basic dye (hematoxylin). The accompanying plate shows the two types of cells in the resting condition and in different stages of mitosis.

It is of interest that these cells have been found only in the 5 mm. larvae of my collection. These particular 5 mm. larvae were procured at Naples by Professor Stockard in the spring of 1910. They were fixed at the time of collection in picro-acetic and preserved in 80 per cent alcohol. I received them in the fall of 1914, sectioned and stained them in hematoxylin and eosin. The remaining specimens of my collection, which comprise developmental stages ranging from the segmentation sphere up to an including a transforming larva and the adult, have been kindly supplied to me by Professor Gage. They were collected from the waters in the neighborhood of Ithaca, N. Y. Unfortunately, I do not have any 5 mm. larvae of the American species so am unable to say whether these cells are peculiar to the European species and to this age of larvae. Several specimens of these 5 mm. larvae show cells with nuclei taking the acid stain. They are not equally numerous nor do they take the stain equally well in all cases. In one specimen, for instance, such cells are difficult to find while in others they are distinct and plentiful. Embryos in which these cells are prominent may show more than half of the blood cells containing nuclei in which the chromatin has absorbed the acid dye.

Cells have been found in which both kinds of chromatin are present. Figure 7 represents such a cell in the resting state. Two lumps of chromatin in the central part of the nucleus have taken the basic stain while the chromatin at the periphery is stained with the acid dye. Heidenhain ('07) has shown a chromatolytic nucleus which somewhat resembles this, but in which the acid-staining chromatin was in the central part of the nucleus while the basic-staining chromatin was at the periphery. Figures 5 and 6 represent cells with two kinds of chromatin in the process of mitosis. Figure 6 shows that a small part of the acid-staining chromatin has apparently been taken over with the basic-staining group. Figure 5 represents the separation of the two kinds of chromosomes in the daughter nuclei which appears to have been complete. The great majority of these cells with acid-staining chromatin, however, are pure in regard to their staining reaction. Figures 1, 4, 8, 9 and 10 show nuclei containing no granule or any other part which absorbs the basic dye.

Stockard ('06) confirmed the observations of Schniewind-Theis ('97) in which it was shown that some nuclei in the deeper layers of actively secreting nectar glands of Vicia faba take the plasma stain. In the living gland some rows of cells have a blue while others have a red coloration. By introducing alkaline and acid fluids to sections of the living gland, Stockard found that these cells responded to the fluids in the same way that litmus does to alkalies and acids. This experiment shows quite conclusively that the chemical reaction of the glandular plant cell is not constant during various physiological phases. The staining reaction also indicates that the nuclei apparently respond to the stain according to their physiological state. The stain used was Auerbach's (methyl green and acid fuchsin) which gives a delicate differentiation of the acid and basic qualities. It was determined in these investigations that materials were formed by the nucleus and passed out into the cytoplasm, the cytoplasm in such cases finally accumulating enough of the nuclear products to stain with the nuclear dye. Further, when the secreting activities of the nucleus had apparently been spent the nucleus stained with the plasma stain. These reactions occurred only in vegetative cells, the dividing cell always contained chromatin which stained in the normal way with the basic dye. In these studies the tissues had been carefully fixed with neutral fluids so as to preserve the chemical reaction of the living cell. The fixation used in my specimens, as was pointed out above, was an acid fluid, yet the differences in the reactions of the cells and portions of some nuclei were sufficient to maintain their character and to respond to the ordinary hematoxylin-eosin stain in the peculiar ways shown in plate 1.

The presence in the same section of the lamprey larva of cells with acidophilic nuclei together with cells which stain in the normal way, and the fact that both kinds of chromatin are present in a single cell, make it difficult to give any other interpretation of these reactions than that they represent the result of physiological changes which occurred during life.

As far as I can ascertain this is the first account of a case where the chromosomes in a dividing cell have definitely taken the acid stain.

LITERATURE CITED

HEIDENHAIN 1907 In Bardeleben's Handbuch der Anatomie Des Menschen.

SCHNIEWIND-THIES, J. 1897 Beiträge zur Kenntniss der Septalnectarien, Jena (cited from Stockard (1906)).

STOCKARD, C. R. 1906 Cytological changes accompanying secretion in the nectar-glands of Vicia faba. Bull. of Torrey Bot. Club, vol. 33, pp. 247–262.

PLATE 1

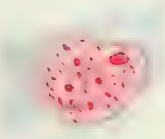
EXPLANATION OF FIGURES

All figures were drawn with the aid of the camera lucida to the same scale of magnification (1/12 oil immersion objective, compensating occular No.12). Higgins' carmine and true blue inks were used in reproducing the colors of the stained specimens.

Figures 1, 2 and 5 are mesenchyma cells; all other figures represent blood cells.

AN INSTANCE OF ACIDOPHILIC CHROMOSOMES
IVAN E. WALLIN

PLATE 1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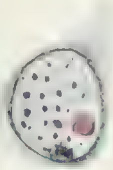
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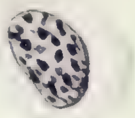
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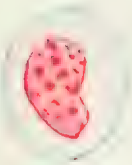
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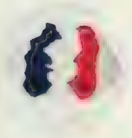
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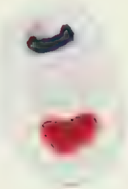
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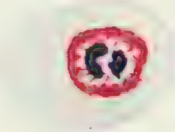
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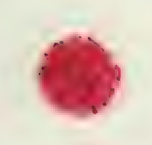
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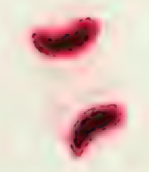
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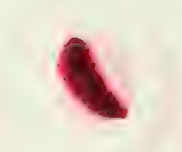
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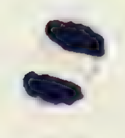
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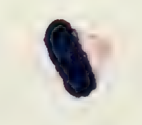
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Reprinted from The Anatomical Record, Vol. 9, No. 1, 1915

2. *The artificial production of spina bifida by means of ultra-violet rays.*

W. M. Baldwin, Department of Anatomy, Cornell Medical College, New York City.

The purpose in presenting this paper is two-fold: first, by reason of the method employed the condition of spina bifida in tadpoles may be produced at will and the level of the bifurcation of the neural tube predetermined; second, the method gives considerable insight into the developmental potentials of the various portions of the ovum, and, in this instance, of the nature and location of the neural tube formative substances. This method consists in the illumination of small surface areas of the fertilized ovum of the frog by means of ultra-violet light of intensity sufficient to kill the area exposed in from 10 to 30 seconds.

It was found that in the undivided ovum the chemical organ-building substances, 'ferments,' or proanlagen, of the neural tube are neither located in any portion of the yolk hemisphere, nor along the equator. These proanlagen occupy a superficial position well up on the surface of the pigmented hemisphere of the egg and attain their definitive extent and position by a process of backward migration or differentiation, keeping pace in this migration with the corresponding shifting of the dorsal lip of the blastopore. These two processes occur synchronously, so that when the rate of the latter is interfered with (as from the presence of an area of dead yolk), the neural tube proanlagen differentiate into half anlagen and then half tubes at approximately their former rate of backward progression, but now along a line corresponding to the equator of the egg and not, as is usual, along the median plane of the egg. The yolk mass is finally completely drawn into the body of the embryo, and as a result the two neural-tube halves are approximated to a greater or less degree. Subsequent fusion of the tube-halves does not, however, occur. Each half-tube by a later shifting of its cell becomes a whole tube.

The experiments add one more fact towards the establishment of the conception of the egg as a composite structure containing, from the first, the organ-building substances of the various body systems, restricted to more or less definitely localized areas in the egg substance (mosaic theory of Roux). Furthermore, the conclusion seems justifiable, tenatively, at least, that the various chemicals such as salts of sodium and of lithium, which have been used by Morgan, Hertwig, Herbst, Jenkinson and others in the production of this malformation, have, at least, produced their effect by acting upon portions of the yolk hemisphere and not necessarily upon the proanlagen restricted to the pigmented hemisphere.

Reprinted from The Anatomical Record, Vol. 9, No. 1, 1915

10. Comparative size of nucleus and cytoplasm in old and regenerating tissues. E. L. Brezee, Cornell University Medical School, New York City. (Introduced by C. R. Stockard.)

The species used for this experiment were Fundulus heteroclitus, tadpoles of Rana sylvatica, and two species of salamander, Diemyctylus viridens adults and Amblystoma punctatum larvae.

Old and regenerating tissue from both adult and larval animals were studied. The tails and arms were cut off about one-third or one-half way from the body. These parts after a few days had regenerated and the organisms were then preservd in Bouin's fluid, sectioned and stained with hämatein and eosin.

Sections were cut in such a plane as would pass through both old and regenerating tissue. Two sections of each specimen were studied and camera lucida drawings made of the nuclear outlines of portions of epithelium and mesenchyme from the old and new tissue of each section. The cell outlines could not be traced as they did not show distinctly enough. In the epithelium the cells were so closely packed that all the substance which was not nucleus could be considered as cytoplasm. In the mesenchyme, however, the cells were so scattered and the intercellular spaces so numerous that the amount of cytoplasm could not be determined in this way; hence no relation between the amount of nuclear material and of cytoplasm could be calculated, but merely the size of nuclei in old and new tissue compared.

The purpose of the experiment was three-fold: (a) to determine whether the nuclei of the old or of the new tissue were larger; (b) to determine in both old and new tissue whether the amounts of nuclear or of cytoplasmic material were greater; and (c) to compare the relative amounts of nuclear and cytoplasmic material in old and new tissue. In each case the areas traced from the old and new tissue of the same section were compared, and the results noted.

(a) By comparing the tracings from each portion of new tissue with the corresponding portion of old it was possible to determine without actual measurement in which the nuclei were larger. The results are shown in table 1.

In the mesenchyme there is a slight advantage in nuclear size shown in the old tissue; this is true also of the epithelium of the larvae but in the adult animals the size of the nuclei of the new epithelium is more often greater.

(b) The actual areas of nuclear and cytoplasmic material were found by the following method: a rectangular portion was outlined and its area found; the mean diameter of each nucleus within this portion was determined and its area found by use of the formula πr^2, letting $\pi =$

3 ½. The sum of these areas gave the total area of the nuclei and by subtracting this from the area of the rectangle, the area of the cytoplasm was found. Then, using the ratio

Area of nucleus : Area of cytoplasm : : 1 : X

the number of parts of cytoplasm to one part of nuclear material was found for each specimen. In the majority of all cases the area of cytoplasm was greater than of nuclear material; i. e., in the ratios X was found to be greater than 1. Table 2 shows the number of cases which were exceptions:

TABLE 1

SPECIES	NUCLEI LARGER IN OLD TISSUE AS COMPARED WITH NEW	EQUAL	NUCLEI LARGER IN NEW TISSUE AS COMPARED WITH OLD
Mesenchyme			
Adult			
Fundulus heteroclitus tail	2 cases	4 cases	4 cases
Diemyctylus tail	31 cases	18 cases	15 cases
Diemyctylus arm	5 cases	3 cases	12 cases
Total	38 cases	25 cases	31 cases
Average	40 per cent	27 per cent	33 per cent
Larval			
Rana sylvatica tail	6 cases	2 cases	2 cases
Amblystoma punctatum tail	10 cases	13 cases	7 cases
Total	16 cases	15 cases	9 cases
Average	40 per cent	37½ per cent	22½ per cent
Adult and larval			
Total	54 cases	40 cases	40 cases
Average	40 per cent	30 per cent	30 per cent
Epithelium			
Adult			
Fundulus heteroclitus tail	7 cases	No cases	3 cases
Diemyctylus tail	21 cases	12 cases	31 cases
Diemyctylus arm	2 cases	2 cases	16 cases
Total	30 cases	14 cases	50 cases
Average	32 per cent	15 per cent	53 per cent
Larval			
Rana sylvatica tail	4 cases	2 cases	4 cases
Ambylstoma punctatum tail	12 cases	9 cases	9 cases
Total	16 cases	11 cases	13 cases
Average	40 per cent	27½ per cent	32½ per cent
Adult and Larval			
Total	46 cases	25 cases	63 cases
Average	34 per cent	19 per cent	47 per cent

The cytoplasmic area was greater than the nuclear in 86 per cent of all cases, the percentage of exceptions being considerably less in the larval than in the adult animals.

TABLE 2

SPECIES	OLD	NEW
Adult		
Fundulus heteroclitus tail	No exceptions	No exceptions
Diemyctylus tail	12 exceptions	9 exceptions
Diemyctylus arm	2 exceptions	7 exceptions
Total	15 per cent	17 per cent
Larval		
Rana sylvatica tail	No exceptions	1 exception
Amblystoma punctatum tail	5 exceptions	2 exceptions
Total	12½ per cent	7½ per cent

Total number of tracings of epithelium	268
Total number of exceptions	38

(c) In a small majority of the cases, 57½ per cent, the number of parts of cytoplasm to one part of nuclear material was found to be greater in the old tissue than in the new. Table 3 shows the summary of the ratios as found for each pair of tracings, the numbers representing X in the ratio

TABLE 3

Summary of tables of areas of cytoplasm to areas of nuclei

NUMBER OF SECTIONS STUDIED	AVERAGE OF PARTS OF CYTOPLASM TO 1 PART NUCLEUS		
	Old	New	
Adult			
10	6.041	6.533	Fundulus heteroclitus tail
64	1.381	1.302	Diemyctylus tail
20	1.428	1.204	Diemyctylus arm
Larval			
10	2.694	2.266	Rana sylvatica tail
30	1.808	1.628	Amblystoma punctatum tail
Total Average.	2.585	2.587	

Area of nucleus : Area of cytoplasm : : 1 : X.

Table 4 shows the percentage of cases for each species in which there is more cytoplasmic material in the old than in the new, as compared with the same amount of nuclear material:

TABLE 4

Adult	*per cent*
Fundulus heteroclitus tail	30
Diemyctylus tail	55
Diemyctylus arm	75
Average	53⅓

TABLE 4—Continued.

Larval	
Rana sylvatica tail	70
Amblystoma punctatum tail	57
Average	$63\frac{1}{2}$
Total Average	$57\frac{1}{2}$

Summary: From the total number of 536 tracings in this experiment the following conclusions may be drawn: (a) In the mesenchyme of both larval and adult animals and in the epithelium of the larval animals the nuclei are larger in the old tissue than in the new; but in the epithelium of the adult animals the nuclei are larger in the new tissue. (b) In 86 per cent of the tracings of epithelium the cytoplasmic area is greater than the nuclear; the larval tissues show fewer exceptions to this rule than the adult. (c) The amount of cytoplasmic material as compared with nuclear is found to be slightly greater in the old than in the new tissue in $57\frac{1}{2}$ per cent of the cases.

In general then it would seem that in the epithelium of the larval animal the whole cell in the old tissue is larger than the cell in the regenerating tissue, the cytoplasm, however, being larger in greater proportion than the nucleus. In the epithelium of the adult animal the nucleus of the old cell is smaller than that of the cell in the regenerating tissue but the amount of cytoplasm is greater per nuclear area.

On the Nature and Formation of the Fertilization Membrane of the Echinoderm Egg.

(Contribution from the Anatomical Department of the Cornell University Medical College and the U.S. Bureau of Fisheries Laboratories at Wood Hole, Mass.)

With 1 figure in text.

(Eingegangen am 3. Februar 1914.)

Many investigators have observed the formation of a sharp line around the echinoderm egg shortly after fertilization. This line is usually supposed to be the optical section of a membrane, the so-called "fertilization membrane". Some observers, notably HARVEY[1] suppose the membrane to be present before fertilization, but to be so closely applied to the egg as to be almost invisible, and after fertilization to be raised from the surface of the egg by the accumulation of a perivitelline substance between the membrane and the egg. LOEB supposed this perivitelline substance to contain a colloid which absorbs water and swells, this pushing out the fertilization membrane. He describes the perivitelline substance as exuding from the egg in droplets, so that one might consider it a colloidal solution. However, it seems to be a "gel" since it assumes a striated appearance, when put under tension; and the egg does not sink or rise in it but maintains a fixed position, as I have determined by observing the egg horizontally in a specially constructed chamber.

KITE[2] attempted to disprove the existence of a fertilization membrane. According to his observations, the unfertilized egg is surrounded by a hyaline layer which he calls the vitelline membrane. He claims that fertilization results in a swelling or increase in thickness of this vitelline membrane, and that what appears to be the fertilization membrane is really the outer surface of a thick membrane, the swollen vitelline membrane. It appears to me that Kite is merely applying new terms to structures which were considered by previous observers, unless he can show that the swollen vitelline membrane is homogeneous. If the outer surface film of this swollen vitelline membrane is more rigid than its interior, then it represents a distinct structure and we are justified in applying the term fertilization membrane to it. The following evidence seems to show that the fertilization membrane is really a structure.

---

[1] Jour. Exper. Zool., VIII, p. 365, 1910.

[2] Science, n. s. 36, p. 562, 1912.

1. In normal development, the fertilization membrane does not sink into the cleavage furrows as the hyaline plasma layer and perivitelline substance does (Arbacia).

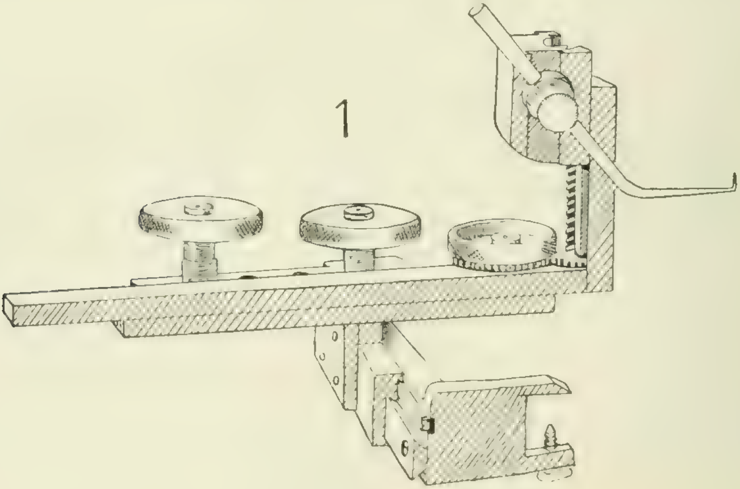

Fig. 1.

„Mechanical hand" used to hold a glass needle or pipette in fine work under the microscope (first described by McClendon; Biological Bulletin, XII, p. 141, 1907). The glass needle shows two bends but usually a glass rod held horizontally and with the tip drawn vertically to a point was used. The egg was in a very thin hanging drop in a damp chamber. The apparatus may be clamped on either side of the microscope stage, preferably on the right, or two may be used simultaneously. Made by Spencer Lens Co., Buffalo, N.Y.

2. Cilia develop before the disappearance of the fertilization membrane and beat actively in the space beneath the fertilization membrane (Arbacia and Asterias).

3. I have manipulated the fertilization membrane of the Arbacia egg with glass needles and punctured it and removed it from the egg and it appears to offer more resistance than the perivitelline substance to the needle (see fig. 1). The egg may be forced out of the fertilization

membrane by means of the electric current[1] and the membrane remains spherical and of large size as though it had considerable rigidity[2].

It seems clear that the old view of the existence of a fertilization membrane is pretty near the truth, and we will now consider its mode of origin.

The egg of the sea urchin, starfish and perhaps other echinoderms, as it comes from the ovary is surrounded by a hyaline, radially striated layer which swells in sea water so that the striations disappear, and it becomes invisible. KITE calls this the egg jelly, but it had received many names previously, i. e. chorion, zona pellucida, jelly, mucus etc. It seems to have the physical properties and solubilities of mucus. There are a number of mucins, and the one derived from frog's egg jelly differs chemically from any known mucin from mammals. We will use the word mucus (without attaching any chemical significance to it) rather than jelly, since the perivitelline substance seems also to be a jelly.

The egg of Toxopneustes variegatus, from which the mucus has been removed, does not show a fertilization membrane, although it segments after fertilization[3]. In an electric current the mucus and egg move toward the anode and the perivitelline jelly toward the cathode. This observation has been confirmed last summer, and shows that the mucus is charged negatively and the perivitelline colloid positively. Since oppositely charged colloids precipitate one another, I advanced the hypothesis in 1911 that the fertilization membrane is a precipitation at the contact of the mucus with the perivitelline colloid, and for that reason removal of the mucus prevents the formation of the membrane[4].

The mucus may be removed from Toxopneustes eggs by washing for an hour or so in large quantities of the sea water near Key West, Florida (which is hyperalkaline as compared with Woods Hole sea water). The mucus is more difficult to remove from Arbacia eggs at Woods Hole, but I have succeeded in doing so by each of the following methods.

1. Rapid centrifuging in haematokrit tubes two or three times, the whole operation taking but a few minutes. The eggs are suspended in fresh sea water each time and the centrifuge started with a sudden jerk.

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[1] McCLENDON, Amer. Jour. Physiol., XXVII, p. 253, 1910.

[2] For apparatus see McCLENDON, On the Electric Charge of Protoplasm and Other Substances in Living Cells, this heft, fig. A.

[3] McCLENDON, Amer. Jour. Physiol., XXVII, p. 240, 1910.

[4] McCLENDON, Science, XXXIII, p. 387, 1912.

2. Washing not more than twenty-four hours in large quantities of sea water in rotating flask filled and closed so as to exclude air bubbles, since the latter injure the eggs.

3. Washing less than 2 ccm of eggs in 10 litres of sea water agitated with an automatic glass stirring rod not more than twenty-four hours. Controls showed that mere standing in sea water during the time required for these operations did not remove the mucus or prevent the appearance of a fertilization membrane.

The presence or absence of the mucus was determined in each case by at least two of the following methods:

First, observing the eggs in a single layer in Chinese or India ink suspension in sea water.

Second, staining the mucus with neutral red, mythylene blue or toluidin blue. Continuous observation is necessary since the dye first stains the outer surface of the mucus, but finally stains all of it and causes it to contract so close to the egg as to be indistinguishable from the egg.

Third, the massing of spermatozoa which become entangled in the mucus[1].

In every case in which the mucus was completely removed before fertilization, no fertilization membrane could be observed after sperm were added, nevertheless the eggs segmented. The same results were obtained by ELDER[2] on a California sea urchin, so we now have uniform results on sea urchins in Florida, Woods Hole and California.

It might be objected that the eggs were injured by removal of the mucus and this injury prevented the formation of fertilization membranes. Such an objection cannot be answered directly, but ELDER and I used several methods for removing the mucus and the eggs were not injured so as to prevent segmentation.

If my hypothesis of the formation of the fertilization membrane is correct, neither the fertilization membrane nor the perivitelline substance should be found on the surface of the unfertilized egg.

I failed to find a membrane on the surface of the unfertilized egg that resisted puncture by a glass needle so much as the fertilization membrane did. I have frequently observed a spermatozoan wriggle through the mucus until its head touched the fertilization membrane, when its progress was stopped, although its tail continued to vibrate as vigorously as before. Previous observers have found that unfertilized eggs are more easily broken up than eggs with membranes by shaking with powdered glass or sand.

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[1] ELDER in the paper cited below claims that the mucus attracts the spermatazoa but apparently it merely mechanically entangles them as stated by F. LILLIE. I removed the mucus from Arbacia eggs and washed them repeatedly for ten days. During all this time they gave out a sperm attracting substance which may be merely CO_2, since CO_2 attracts them.

[2] Arch. f. Entw.-Mech. XXXV, p. 195, 1913.

These observations show that the characteristically tough fertilization membrane does not surround the unfertilized egg.

The process of "membrane formation" was observed in eggs of Arbacia and Asterias with Zeiss 2 mm, homogen. imm. apochromat. obj. and compens. oc. 12 and lowers powers by daylight; also with a high power dry lens with the ultramicroscope. It was found that certain appearances might be due to the curvature of the surface of the egg. An air bubble the same size as the egg appears to be bounded by a sharp circular line, but both outside and inside this circle are a series of concentric circular lines which grow fainter in proportion to their distance from the sharp line. Let us call them pseudo-lines. The surface of the unfertilized egg has a border zone free from granules and bounded externally by a sharp line. Outside this sharp line appears a series of pseudo-lines the same distance apart as the pseudo-lines around the air bubble. The greatest number I have seen by direct daylight is three, but with the ultramicroscope five are very distinct. The fact that they are variable in number strengthens the view that they are false visual impressions. But why do we not see pseudo-lines inside the sharp line? The answer seems clear. The line of separation of the hyaline border zone and granular interior is in the position that should be occupied by the first pseudo-line and is probably intensified by it, and the other pseudo-lines are obscured by the granular cytoplasm.

In observing fertilized eggs by all the above methods the fertilization membrane at some foci looks like the surface of the air bubble described above, but at a certain focus (which I think is the true focus, the others being out of focus) the sharp line appears double, the two sharp lines apparently forming the inner and outer boundaries of the membrane. I have repeatedly observed eggs just after fertilization when the membrane was separated only over a small part of the surface, and attempted to trace the two sharp lines of the membrane into their relation with the egg surface over which no membrane had yet appeared. One might in this way hope to see whether the membrane arises from the egg cortex or from the mucus, or from both. The pseudo-lines seen with high powers and the obvious difficulties with low powers prevent a positive answer to this question, but it seems clear that the whole granule free cortex of the egg is not lifted off to form the fertilization membrane, for immediately after the fertilization membrane appears the granule-free cortex is as thick as before. The granule-free zone appears to thicken later to form the hyaline plasma layer.

J. Loeb supposes the perivitelline colloids to be set free at the surface of the egg after fertilization, i. e. not to form the most superficial layer of the egg before fertilization. Such a view seems necessary if my hypothesis is correct, but such a process has not been demonstrated to every one's satisfaction, and my hypothesis remains a hypothesis.

Summary.

If the mucus layer (jelly, chorion, zona pellucida) is removed from the sea urchin's egg before fertilization, no fertilization membrane appears, although the egg segments. No membrane of the toughness that characterizes the fully developed fertilization membrane exists on the surface of the unfertilized egg.

Zusammenfassung.

Wenn die Schleimschicht (Gallerte, Chorion, Zona pellucida) vor der Befruchtung von dem Seeigelei entfernt wird, zeigt sich keine Befruchtungsmembran, obgleich sich das Ei teilt. Es besteht keine Membran von der Zähigkeit, die die voll entwickelte Befruchtungsmembran charakterisiert, an der Oberfläche des nicht-befruchteten Eies.

On the Electric Charge of the Protoplasm and other Substances in Living Cells.

(Contribution from the Anatomical Department of Cornell University Medical College.)

With 1 figure in text.

(Eingegangen am 3. Februar 1914.)

A number of investigators have observed that when an electric current is passed through a suspension of animal cells, it carries them passively toward the anode, indicating that the cells are charged negatively. This may be explained by the hypothesis that the plasma membrane of the cell is permeable only to kations, so that after the loss of some kations by diffusion, the excess of contained anions gives the cell a negative charge. Another hypothesis is that all of the protoplasm is charged negatively.

Electric currents were passed through animal and plant cells, and many substances of or in the protoplasm migrated toward the anode, showing that they were charged negatively. It appeared, also, that the denser substances, such as chromatin granules, yolk platelets and mitotic figures migrated faster than the other substances. Though this tended toward the belief that all of the cell contents, except water, are charged negatively, the slowness with which hyaline parts of the protoplasm of some cells migrated prevented a determination of their electric charge. The object of the present paper is to speculate on the cause of this negativity of protoplasm.

It is generally recognized that proteids, such as egg albumin, are anodic (or negative) in alkaline solution and kathodic (or positive) in acid solution, because they are amphoteric electrolytes and dissociate according to the formula:

$$NaR = Na^{+} + R^{-} \text{ (Alkali albuminate)}$$

$$\text{and } RCl = R^{+} + Cl^{-} \text{ (Acid albumin).}$$

We might postulate that the interior of the cell is alkaline and that the superficial protoplasm dissociates positive ions, such as Na^+, into the surrounding medium, thus making the cell negative, or that it absorbs OH^- ions.

It has been commonly supposed that the reaction of the cells of the body is maintained constant by osmotic interchange with the blood, and the blood was formerly considered alkaline. But the blood has been shown to be neutral, and many cells appear to be impermeable to many substances that might affect their reaction. Furthermore, there seems to be no direct method of testing the reaction of the cell interior unless we rely on indicators. Those indicators taken up by living cells, as neutral red, are immediately bound up in some manner with colloids, and it seems unwise to regard them as infallible.

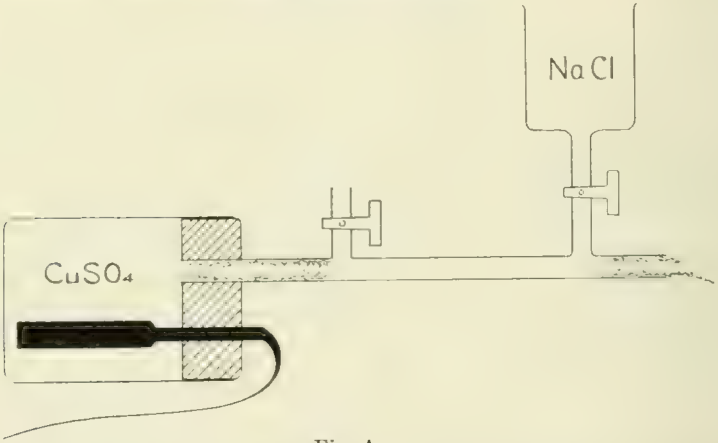

Fig. A.

One of the two electrodes used in passing the 116 volt direct current through cells placed between slide and cover glass, under the microscope. The non-polarizable electrode is connected with a glass tube filled with Na Cl solution (usually about 1 %) a plug of cotton retards diffusion between $CuSO_4$ solution and NaCl. Another plug of cotton closes the end of the tube and is pulled out to a point to communicate with the preparation under the microscope. By opening the two stops cocks any $CuSO_4$ which has diffused into the tube may be washed out, by a flow of solution *toward the electrode.* The amount of current may be changed by the concentration of salt in the solution.

If protoplasm is negative, because it is saturated with an alkaline solution, the supposition would easily follow that treatment with acid would make it positive, just as egg albumin is made positive. To test this I found plant cells that are permeable to acid while yet alive. But these cells are easily injured by the electric current, and the presence of the acid seemed to make them more sensitive to the electric current, so that they died before any appreciable amount of protoplasm had been displaced.

Although these experiments have failed in regard to observations on the living "protoplasm", interesting results were obtained on the electric convection of substances in the vacuoles of living plant cells. The vacuoles of some plant cells contain a colored substance, anthocyan in (colloidal?) solution.

Although very little chemical significance is attached to this term, anthocyan is an acid-alkali indicator. It has been suggested that in some cells, such as those of the nectar glands of Vicia faba, the anthocyan changes from blue to red and vice versa during the functional activity of the cell. These cells and also those of red beet and red cabbage are permeable to acids and alkalis, as indicated by the change in the color of the anthocyan. In cells of the red cabbage the anthocyan is normally colored red and is kathodic, but if ammonia is added it becomes blue-green and is anodic. This experiment is a very tedious one because the cells are easily injured by the current. Notwithstanding this disadvantage, I succeeded in 1910 in observing the movement of red anthocyan toward the kathode and blue-green anthocyan toward the anode in cells, before any signs of death or injury occurred. These experiments have recently been repeated many times to exclude any possibility of error. We may conclude, therefore, that the anthocyan is an amphoteric electrolyte and that its red color and kathodic migration in any cell denotes acidity of the cell sap in which it is dissolved.

If the protoplasm behaves as the anthocyan, many plant cells are probably acid and kathodic, since the anthocyan is red. The protoplasm of cells in growing root tips appears to be anodic. The protoplasm of old cells forms a thin layer that sticks tightly against the cell wall and cannot easily be dislodged by the electric current. This cell wall also interferes with the interpretation of results on the electric convection of the whole cell, since the whole cell would have the charge of the superficial layer, in this case, a cellulose cell wall. THORNTON[1] observed that plant cells differed from animal cells in being usually kathodic. Some of these cells were motile and might have shown galvanotropism, but others were not. Even non-motile naked cells might be kathodic on account of adsorption of positive ions. HÖBER[2] observed that the addition of small amounts of H^+, Ag^+, Cu^{++}, or Fe^{+++}, made erythrocytes and yeast kathodic, and very probably the heavy metals were adsorbed. HÖBER concludes[3] that CO_2 makes erythroyctes kathodic by increasing their permeability to anions since they become kathodic, only in an anion-poor solution (2% NaCl) and not in an anion-rich solution (8% NaCl). All of these observations show that in such cases we should not consider the electric convection of the whole cell as indicating the electric charge of the whole protoplasm, but only of the superficial layer of the cell or cell wall.

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[1] THORNTON, Proc. Royal Soc., B., Vol. 82, p. 638, 1910.

[2] Physikalische Chemie der Zelle im Gewebe, S. 388, 1911.

[3] Ibid. p. 495.

Summary.

The anthocyan in the vacuoles of living cells of red cabbage is red and is kathodic (electrically positive), if alkali be added to the medium the anthocyan becomes blue and anodic (electrically negative) before the death of the cell. Therefore, the anthocyan seems to be an amphoteric electrolyte like egg albumin.

It seems possible that the reason that protoplasm is anodic is because it is alkaline, but this is not demonstrated.

Zusammenfassung.

Das Anthocyan in den Vakuolen der lebenden Zellen von Rotkraut ist rot und ist kathodisch (elektrisch positiv); wenn man Alkali zu dem Medium fügt, wird das Anthocyan vor dem Tode der Zellen blau und anodisch (elektrisch negativ). Daher scheint das Anthocyan ein amphoterer Elektrolyt, wie Eiereiweiß, zu sein.

Es scheint möglich, daß der Grund, weshalb Protoplasma anodisch ist, darin liegt, daß es alkalisch ist, aber das ist nicht bewiesen worden.

On the Absorption of Water through the Skin of a Frog.

(Contribution from the Anatomical Department of Cornell University
Medical College.)

(Eingegangen am 4. Februar 1914.)

The work of Overton seemed to show very clearly that the whole frog, or the excised muscle, when placed in water or a hypotonic solution, absorbs water, and that the driving force of this absorption is the osmotic pressure of the tissue and cell fluids. It was rather surprising, therefore, that Martin Fischer should imagine that the driving force of this absorption is the swelling of colloids, and to construct on this hypothesis theories of edema and nephritis. I do not wish to consider either edema or nephritis, but merely to consider wheter any quantitative relations of the absorption of water by the frog or through the frogs skin are inexplicable on the theory of osmotic pressure.

Fischer ligated the leg of the frog at the knee so as to cut off the circulation of blood and lymph, and so long as the leg remained in water, it absorbed water. Obviously it made no difference whether the leg was amputated or remained attached to a live frog. My explanation of this is that the leg, and all other parts, of the frog is constantly absorbing water while immersed, but that this water is constantly being eliminated by the kidneys so that the leg retains its normal water content. But as soon as communication with the kidneys is interrupted by a ligature at the knee (thus stopping the blood and lymph flow) the water absorbed begins to accumulate in the leg. The same is probably true of the excised muscle, eyeball or any other part, while living, and perhaps for some time after death of the cells.

It was not practicable to measure the volume of blood flowing to the leg and subtract it from the volume of blood (and lymph) leaving the leg in order to see whether the leg with a circulation absorbs water at the same rate as the leg that is ligated. However, by making the assumption that water is absorbed through all regions of the skin of the immersed frog at the same rate per unit area, a method of experimentation was found.

The skin area of a leopard frog (*Rana pipiens*) exclusive of the head was measured by pasting pieces of waxed cross section paper over it and then computing the number of squares on the paper. This may seem a crude method but it was the most accurate that was found. By this means it was determined that the ratio of the whole skin area (exclusive of the head) to the area of the two hind legs (and feet) below the knees, was 3,495. In a wood frog (*Rana sylvatica*) this was found to be 3,497. Since the errors of measurement are probably too great to make the third decimal place of any use, we will consider this ratio as 3,5.

Two leopard frogs of the same weight were selected. One was weighed and then fixed in an apparatus so that the urine was collected, and the frog kept immersed up to the head in water. It could drink no water and it breathed a damp atmosphere, so that there could be no evaporation from the lungs. The two legs of the other leopard frog were ligated just above the knee, amputated immediately above the ligature and weighed, and then immersed in water. At the end of twenty hours the frog (+ urine) was weighed and the two legs were weighed. Taking the average results of three experiments the frog had increased 11 grams, the legs had increased 1,1 gram. The ratio is 10 as compared with 3,5 for the ratio of skin areas. In other words, the absorption of water through the skin of the normal frog was nearly three times as rapid as the absorption of water through the skin of the excised legs per unit area. On the osmotic theory this may be explained by the fact that the osmotic pressure throughout the normal frog was maintained equal to a 0,7% solution of NaCl by the action of the circulation and kidneys, whereas, the osmotic pressure in the skin of the amputated leg was rapidly lowered by the mixture of the first water absorbed with its fluids, and hence the rate of absorption of water was rapidly reduced.

In an experiment with wood frogs lasting five hours, the frog increased in weight 0.67 grams and the two hind legs 0,15 grams. In this case the ratio was 4,5 or not very much more than the ratio of skin areas, 3,5. It seems probable that if the time had been very short, the ratio would have been the same, but owing to errors concerned with weighing, this was not attempted, as it was feared that the increase in weight would not be very much larger than the errors. It should be noted that every effort was made to reduce errors — the skin was wiped in the same manner each time and the frogs and legs were weighed in closed glass vessels to prevent evaporation.

The effect of circulation of the contained fluids in the leg was shown in the following manner: It was determined that in 0.7% NaCl the leg of a wood frog neither gained nor lost weight.

The two legs of a wood frog were amputated above the knee and one was ligated just below the amputation. The other was carefully skinned and the leg taken out without injuring the skin. The skin was then filled with 0.7% NaCl solution and ligated at the same level as the other leg and the results given in the following table:

	Increase in weight in grams	
Hrs.	leg	skin
1	0,7	0,07
3	0,155	0,21
6	0,22	0,47

It is seen that the absorption of water by the leg, and the skin filled with salt solution, continued to be the same for one hour, during which time the water absorbed was probably all held in the skin itself. After this, however, the skin filled with NaCl solution absorbed water faster. The various membranes and colloidal "gels" in the frogs leg retarded the inward diffusion of the water, so that it was retained in the superficial layers and so diluted the fluids as to reduce the osmotic pressure and retard further diffusion. The water absorbed into the NaCl solution was rapidly distributed by convection currents and free diffusion, throughout the whole solution, and the osmotic pressure was but slowly lowered.

Since the skin of the frog is electrically polarized (being negative on the outer surface) and since electric polarization has been claimed to cause polarized or one-sided diffusion, experiments were set up to compare the rate of osmosis through frogs skin in opposite directions.

The legs of a wood frog were skinned and one of the skins turned inside out. The two skins were filled with 0.7% NaCl solution and immersed in water. If there was any difference in the increase in weight of the two skins it was too small to be detected.

In a second experiment a piece of wood frog's skin was stretched tight as a diaphragm separating water from 0,7% NaCl solution and the rate of osmosis determined. Water was first placed on the "outside" and 0.7% NaCl on the "inside". After half an hour water was placed on the "inside" and 0.7% NaCl on the "outside" and the osmosis continued another half hour. No difference in the rate of osmosis in the two directions was detected.

That electric polarization and other factors that are dependent on the life of the cells in the skin affect diffusion of some substances I do not doubt, but nearly, if not quite 95% of the osmosis of water in these experiments is fully accounted for by the osmotic pressure of the fluids in and between the cells of the tissues.

Contrary to FISCHER, the effect of stoppage of the circulation is to decrease rather than increase the absorption of water. Only after the tissue has long been dead does an acidosis, autolysis or increase in permeability increase the absorption of water.

Summary.

The absorption of water by a frog's leg in which the circulation has been stopped is the result of the osmotic pressure of the fluids in and between the cells of the tissues.

Zusammenfassung.

Die Wasserabsorption eines Froschschenkels, in welchem die Zirkulation gehemmt wurde, ist das Resultat des osmotischen Druckes der Flüssigkeiten in und zwischen den Gewebezellen.

Postscript: This paper was read Apr. 16, 1913: Proc. Soc, Experimental Biology and Medicine, X, 125. Since then a paper by S. S. Maxwell: Amer. Journ. Physiol., XXXII, 286, appeared, on the same subject which does not, however, disagree with my conclusions. I found the freezing point of the blood serum of the leopard frog (*Rana pipiens*) lower than that of a 0,65% NaCl solution, and the same was found true of *Rana esculenta* by BOTAZZI.

VII.

EXPERIMENTS ON THE PERMEABILITY OF CELLS.

BY J. F. MCCLENDON,
Instructor in Physiology, University of Minnesota Medical School.

Three text-figures.

EXPERIMENTS ON THE PERMEABILITY OF CELLS.

By J. F. McClendon.

One of the most important steps in the analysis of life was the discovery of oxygen. Ever since that time it has been known that animals absorb free oxygen and give it out in a combined form.

The discovery of enzymes has given a clue to the rapidity of oxidation within cells; but enzymes are always present in living substance, and yet oxidation may vary from almost zero in certain eggs and seeds during rest to a high rate during activity. We know that oxidation within muscle is greatly increased during contraction, yet hardly anyone would suppose this to be due to the sudden increase of oxidases. Loeb, however, did use this explanation for the increased oxidation of the egg on fertilization, by suggesting that the sperm carries an oxidase into the egg. Certainly the sperm contains an oxidase, as does every other cell, but the sperm is minute in comparison with the egg, and no one has found any indication of an unusual amount of oxidase in the sperm. Furthermore, the egg may be made to divide by artificial means without the introduction of any enzyme.

An organism may be caused to absorb more oxygen by any one of a number of different stimuli. But what is stimulation? What is the first change induced in the organism or excised organ by all stimuli?

Pfeffer showed that stimulation of sensitive plants causes a change in permeability of certain cells (of the pulvinus). This change in permeability causes a change in turgor (internal osmotic pressure) and movement results. The question arises: what relation does this bear to oxidation? According to Ralph Lillie oxidation is suppressed in the cell by the accumulation of waste products to which the resting cell is impermeable. Stimulation increases the permeability of the cell to them and consequently oxidation is increased. Lillie maintained that on fertilization the egg became more permeable, and linked all subsequent phenomena with this change. Not finding any other explanation of the stimulating action of the sperm, I attempted an exact study of the permeability of the egg.

EXPERIMENTAL.

Three methods of procedure were followed: (1) the use of cell masses as partitions (on eggs of *Lytechinus*); (2) the use of quantities of eggs suspended in a liquid medium (on eggs of *Fundulus*); (3) experiments on individual eggs (of *Arbacia*).

The first of these methods was tried at the Tortugas laboratory on the eggs of the sea-urchin *Lytechinus* (*Toxopneustes*) *variegatus*. Advantage was taken of the fact that the electrolytes of the eggs and the medium dissociate into ions which bear electric charges, and therefore their movement through the eggs could be detected, with a high degree of accuracy, by the electric conductivity method of Kohlrausch.[1]

Several bushels of sea-urchins were collected each day and the eggs of the ripe females placed in large dishes of sea-water. The mucus egg membranes (jelly, zona pellucida) were washed off and the eggs were introduced into a conductivity vessel made especially for the purpose (fig. 1). The conductivity vessel was placed in the centrifuge and the eggs were precipitated until they were closely packed together. By microscopic examination it was found that only a trace of sea-water was left in the spaces between the eggs (fig. 2).

By raising the electrodes above the level of the eggs they came to lie in the supernatant sea-water that had been pressed out from between the eggs. In this way the conductivity of sea-water and egg-mass could be measured separately within two minutes. The conductivity of the egg-mass, when moderately centrifuged, was about one-twentieth of that of the sea-water, indicating that the conductivity of the egg itself must be almost nil.

The conductivity of the egg-mass was greatly affected by the degree of packing. However, the vessel was so long and narrow that the packing of the eggs could be recorded accurately by marking their upper limit on the side of the vessel (fig. 1, *a*). The eggs could then be removed from the conductivity vessel, replaced, and centrifuged to the same level, when the conductivity was found to be the same. In this way I was able to measure the conductivity before and after fertilization and before and after artificial stimulation causing cleavage. It was found that the conductivity increased about one-fourth when the developmental processes began. The sources of error and methods of controlling them are discussed in another paper.[2]

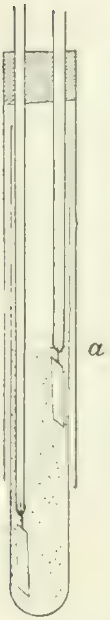

Fig. 1.

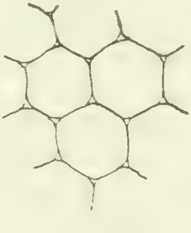

Fig. 2.

One point, however, the elimination of the fertilization membrane, needs further consideration. Soon after the egg is fertilized, a fine line appears close around it and gradually expands until it forms a circle, widely separated from the egg. Almost every one (except Kite) supposes this to represent a spherical membrane, the "fertilization membrane." Before fertilization the egg is covered by a thick transparent jelly or mucus layer, called the chorion or zona pellucida by some authors. I stained this jelly with basic dyes (neutral red and methylene blue). These dyes coagulate the jelly, causing it to become membranous.

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[1] Ostwald-Luther. Messungen.

[2] McClendon. Amer. Jour. Physiol., vol. 27, p. 240.

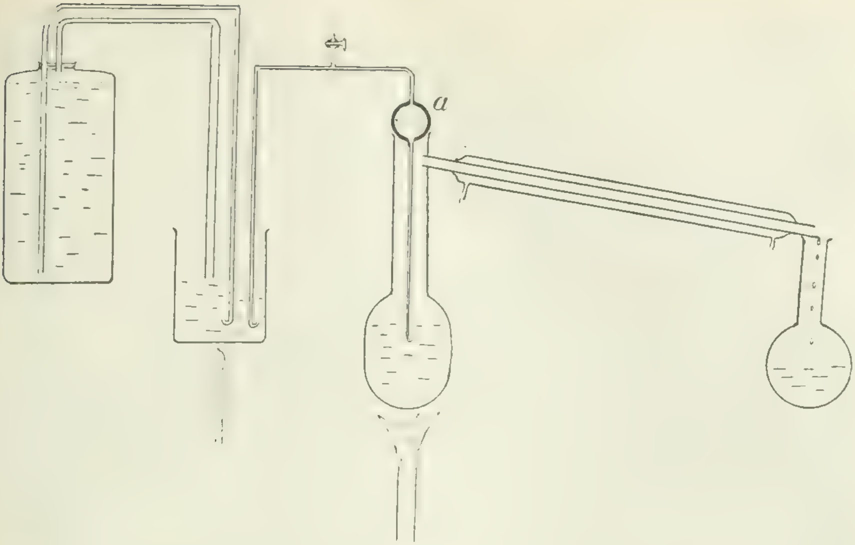

FIG. 3.

In these experiments all toxic substances were eliminated from the water by redistilling it in the apparatus shown in figure 3. The stock bottle (to the left) is filled with distilled water, which siphons over into the beaker and keeps the latter filled up to the mouth of the wide tube. A small flame under the beaker boils the water and drives out all dissolved gases (since the water contains no salts to hold back CO_2, and no non-volatile substances to liberate ammonia). The boiling water from the beaker siphons over into the Kjeldahl flask over a large flame. If the siphon be drawn out into a fairly small opening the Kjeldahl flask may be set lower than the beaker, as in the figure; but this has the disadvantage that when the still is cooled the Kjeldahl flask fills up to the neck and makes it inconvenient to start again. The siphon has a bulb blown on it at *a*, to stop the mouth of the flask. It is not necessary that this stopper be ground in, provided it fits fairly snug, as water condenses on the stopper and seals the crack. A hole is blown in the side of the neck of the flask to admit the condensing tube. This hole may be reamed out with the end of the tube itself, while the glass is hot, to insure a fairly close fit. The condensing tube is of fused quartz and the only water that collects in the receiving bottle is condensed on the quartz. The cold water dissolves glass very slowly, so that it is permissible to use a glass receiving bottle if the water is distilled fresh each day. Hard glass (Jena or Bohemian) dissolves more slowly but contains heavy metals which are toxic even in the minute quantities that dissolve away in the cold distilled water, even though the glass be previously steamed out. Soft glass liberates traces of sodium, calcium, and potassium, which render the water less toxic, but which, if present in sufficient quantity, may be a source of error in the experiment.

In a previous paper[1] I concluded that the fertilization membrane is the result of a mutual coagulation and precipitation between the jelly and another transparent colloid filling the space between it and the egg. I found that if the jelly be removed from the egg no fertilization membrane can be formed. Elder[2] subsequently made the same observation and came to the same conclusion. No matter what is the nature of the membrane, the fact that it can be suppressed is of importance in my technique, since the presence of the membrane might be considered as a source of error.

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[1] McClendon. Science, vol. 33, p. 387, Mar. 10, 1911. [2] Arch. f. Entwicklungmech., vol. 33, p. 143. 1912.

The increased conductivity of the eggs indicates increased permeability to electrically charged particles, since electrons can not pass in the free state through solutions without very soon attaching themselves to atoms. The particles concerned must be ions, since colloidal particles move slowly and carry little electricity per unit mass, and could hardly cause the great increase in conductivity observed.

This increase in permeability was confirmed by plasmolytic experiments on the eggs of *Arbacia* at Woods Hole.

What ions are concerned in the increase in permeability has not been determined. Experiments which I made on the *Fundulus* egg are suggestive.[1] *Fundulus* eggs (which normally develop in sea-water), when developing in distilled water, do not give up chlorides to the water. If placed in sulphates or nitrates of sea-water metals, they still do not give up chlorides. If placed in distilled water they do not give up magnesium (or calcium). But if placed in sodium salts they lose magnesium (and probably calcium and potassium), and chlorine, because the pure sodium increases the permeability.

A critical review of the literature on permeability is given in a previous paper[2] but I shall here refer to some of it briefly. In the summer of 1910 R. Lillie, Lyon and Shackell, E. N. Harvey, and the author, all working on the sea-urchin's egg, had come to the conclusion that its permeability increased on fertilization. Very shortly there appeared a paper by Loeb[3] in which he made this statement:

> In former papers, especially in a book published a year ago, I pointed out that the process of membrane formation, or a certain alteration of the surface of the egg, is the essential cause of the development of the egg; and I pointed out, also, that this alteration of the surface might increase the permeability of the egg, especially for hydroxyl ions.

I have found one such paper,[4] but it was directed against the view that the cell is impermeable to electrolytes, and gave evidence to show that the egg is more permeable to salts than to sugar. In the book referred to[5] Chapter XIII deals largely with permeability and ends with the following conclusion (on page 118):

> Wir kommen also zu dem Schluss, dass die Hydroxyloinen des Seewassers auch in das unbefruchtete Ei diffundieren, und dass die Membranbildung des Oxydationen im Ei nicht dadurch steigert, dass sie die Durchgängigkeit desselben für Hydroxylionen erhöht, sondern dass sie die für die Entwicklung nötigen Oxidationen auf anderem Wege entfesselt oder möglich macht.

If Loeb has found evidence that the permeability of the egg increases on fertilization, or fatty acid treatment, I would be very glad to have my attention called to it, but it is difficult to find a paper without a reference to the journal in which it is published.

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[1] Amer. Jour. Physiol., vol. 29, p. 295, Appendix II.
[2] McClendon. Biol. Bull., vol. 22, p. 113. 1912.
[3] Loeb, J. Science, n. s., vol. 32, p. 412. Sept. 23, 1910.
[4] Loeb. Univ. of California Publications, Physiol., vol. 3, No. 11, p. 81. 1908.
[5] Loeb. Die Chemische Entwicklungserregung des tierischen Eies. Berlin. 1909.

The degree of permeability is of importance in development. R. Lillie maintains that too great an increase in permeability causes cytolysis of the egg. Gray[1] attempts to explain the abnormality of Echinoderm hybrids by assuming that the foreign sperm produces the wrong degree of permeability and this leads to some of the chromatin becoming pathological and being thrown out of the spindle.

The changes in permeability are probably due to changes in the aggregation state of colloids: either proteids, lipoids, or proteid-lipoid combinations or associations. Many investigators have looked for such changes with the microscope. That some change takes place at the surface of the egg on fertilization has long been known, but opinions differ as to its exact nature. Spherical concentric surfaces, when viewed tangentially with the microscope, cause optical illusions, and the real structure can not be exactly determined without knowledge of the refractive indices of the substances composing it. Kite, however, claims that a thin layer may be observed on the surface of the unfertilized egg and, on fertilization, this layer absorbs water and swells. He claims to have stained it differentially.[2] I do not understand, however, his observations on unstained eggs. He says that this layer which he calls the vitelline membrane becomes more distinct (of different refractive index) from the sea-water on swelling, whereas it is well known that all hydrophile colloids, on swelling, become less distinct and more nearly of the same refractive index as the medium.

The swelling of this layer might cause an increase in permeability, since the swelling of all hydrophile colloids increases their permeability to water-soluble substances. Lillie, however, believes that a reversible increase in permeability takes place in the superficial layer of the cytoplasm (plasma membrane) and J. Gray who has confirmed my determination of the increase in permeability of the egg finds that impermeability returns in 15 minutes, whereas Kite's vitelline membrane remains swollen. I consider Kite's vitelline membrane a new name for a colloid postulated by Loeb.

Mitchell and the author[3] studied experimentally the role of hydroxyl ions in the increase in oxidation of sea-urchin's eggs, which follows fertilization or artificial stimulation. We concluded that the increased permeability allows the hydroxyl ions of the sea-water to penetrate the egg. This increase in hydroxyl ions within the egg might increase oxidation.

CONCLUSIONS.

The permeability of the egg to ions and perhaps some other substances increases on fertilization. The unfertilized egg is perhaps in a dormant condition and the increase in permeability probably allows a rapid interchange with the surrounding medium necessary for activity (development).

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[1] Gray, J. Proceedings of the Cambridge Philosophical Society, vol. 17, p. 1, 1913.
[2] Kite. Science, n. s., vol. 36, p. 562. 1912.
[3] Jour. Biol. Chem., vol. 10, p. 459.

Whereas this supposed significance of permeability has not been proven, the sea-urchin's egg is not an exception. Spores, eggs, seeds, and pupæ are usually inclosed in relatively impermeable membranes. A striking example is the cocoa nut. Whereas an active organism may be partly inclosed in a relatively impermeable skin, great activity is always associated with a rapid interchange with the medium, and this is possible through more permeable portions (lungs, gills, kidneys, gut).

The relation of permeability to oxidation can hardly be determined until more is known about the mechanism of animal oxidations. These seem to depend on structure since complete oxidations cease when structure is completely destroyed. Reference is made only to oxidations resulting in the formation of $CO._2$ Oxidizing enzymes such as tyrosinase, which are independent of structure, do not completely oxidize the substances acted on.

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THE EXISTENCE OF A TYPICAL OESTROUS CYCLE IN THE GUINEA-PIG—WITH A STUDY OF ITS HISTOLOGICAL AND PHYSIOLOGICAL CHANGES

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ONE TEXT FIGURE AND NINE PLATES

1. INTRODUCTION

The existence of a more or less regular and definite oestrous cycle has been recognized in a number of mammals, particularly among the different classes of primates, carnivores, ungulates and insectivores. Yet very little is actually known or understood regarding the oestrous cycles and heat periods of a great many other very common mammals. Strangely enough, our knowledge of the sexual rhythm in the guinea-pig is much confused and not properly understood despite the great number of breeding experiments and the several studies of the sexual conditions which have been performed on this animal.

While conducting an extensive breeding experiment with guinea-pigs for the past several years it has become more and more desirable to know their exact oestrous periods.[1] A careful study of the existing literature bearing on this subject serves merely to produce uncertainty and confusion regarding their ovulation times and heat seasons. The reason for such a lack of knowledge is that these small rodents do not reveal in a very evident manner the existence of their typical sexual rhythm as do many mammals of other classes.

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[1] Throughout this paper we have used the terminology proposed by Heape, Quar. Jour. Mic. Sc., vol. 44, 1900, and adopted by Marshall and others. Anoestrous period or anoestrum, period of rest in the female; prooestrum, the first part of the sexual season; oestrus or oestrum, especial period of desire in the female; metoestrum, the short period when the activity of the generative system subsides and the normal condition is resumed in case conception did not occur; dioestrum, the short period of rest which in some mammals lasts only a few days. Such a short cycle as we shall describe in the guinea-pig consisting of four periods the prooestrum, oestrum, metoestrum and dioestrum is known as a dioestrous cycle.

The guinea-pig never, or only in rare cases, shows an external flow from the vagina, and there is no easily noticeable change in the appearance of the external genital organs during the different periods of sexual activity. The only expression generally observed of the sexual condition or heat period in the female is her willingness to accept the male, and this sign is, of course, only manifested when a male is present and a copulation takes place. The copulation then brings about the disturbing factor of pregnancy and the observation of the return of the heat period is prevented. The practical difficulties in observing successful copulation in these animals makes the study of their sexual conditions still more difficult.

Marshall ('10), in a recent summary has stated the case as follows:

> It is difficult to determine the length of the prooestrum and oestrus in rodents, since the external changes which characterize these conditions are comparatively slight. Heape says that the prooestrum in the rabbit lasts, probably, from one to four days. At this time the vulva tends to become swollen and purple in color, but there is no external bleeding. The same may be said of the rat and the guinea-pig; but, in the experience of the writer, *it is generally impossible to detect the prooestrous condition* in either of these animals with absolute certainty.

It must be recalled here that Marshall has devoted a great deal of study to this subject.

The difficulty in observing signs of heat in the guinea-pig has led a numbers of workers during the past fifty years to a study of the ovaries in order to establish the ovulation cycle. The results of such studies, as we shall point out beyond, are inaccurate and confusing in all cases.

Recognizing the above state of affairs, we determined to ascertain whether by a more minute examination of the genital organs of the female it might not be possible to observe an oestrous cycle. In order to examine the vagina thoroughly we have introduced a small nasal speculum which facilitates a clear view of the interior and a smear is made of any fluid that may be present.

A microscopic study of these vaginal fluids, to be described in the following pages, has shown that the guinea-pig possesses a perfectly regular and typical dioestrous cycle. And further, the surprising fact that the composition of the fluids is exactly comparable to the menstrual fluid taken from so high a mammal as the monkey. Heape, ('99), states that the menstrual fluid of the monkey contains a mucous secretion of the uterine glands, blood corpuscles, particles of stroma and epithelium from the uterus and the vagina and leucocytes. All of these elements are present in the fluid from the vagina of the guinea-pig during heat though the relative amounts differ from those in the monkey and the fluid is rarely sufficiently abundant to be recognized on the vulva.

The great advantage of this simple method of examination for the study of the oestrous cycle in these mammals which show no external signs of heat is evident, and we trust that the method may prove useful to those who find it necessary or desirable to know accurately the sexual periods in animals used for experimental breeding.

Having begun a study of the vaginal smears from guinea-pigs we have been led to a more complete consideration of the uterine changes which alter the composition of these smears, and finally to an investigation of the changes in the ovary and the process of ovulation and corpus luteum formation which accompany the activities on the part of the uterus. The present contribution comprises the results of these investigations.

2. CONSIDERATION OF THE LITERATURE ON OVULATION IN THE GUINEA-PIG

It has been recognized for more than half a century that the guinea-pig comes into heat very quickly after giving birth to a litter of young. This period immediately following parturition has been the starting point for the great majority of studies on the sexual behavior of this animal and it has been demonstrated frequently by such studies that ovulation takes place a few hours after parturition, the female accepting the male at that time. These facts are generally admitted but the most varied opinions prevail regarding the times of the subsequent ovulations, when conception does not occur soon after parturition.

The question whether ovulation in the guinea-pig is spontaneous or dependent upon copulation has often been raised by various workers. The majority are of the opinion that ovulation is, or may be, spontaneous although influenced by copulation, and that there is no definite regularity or typical periodicity in the ovulation cycles.

Bischoff, was one of the oldest advocates of the theory of spontaneous ovulation. In a special paper devoted to the study of this problem in 1844, and later in a study of the development of the guinea-pig ('52), he defended the view that the guinea-pig, like all other mammals, has a spontaneous ovulation. Bischoff states that the mature eggs reach the oviducts through the rupture of the greatly distended Graafian follicles during the first twenty-four hours following parturition. This fact, he points out, had previously been observed and was generally accepted by the earlier investigators with the exception of Schulz, 1829, who failed to recognize a heat period before the fifteenth day after parturition, and sometimes even to the forty-ninth day.

According to Bischoff copulation takes place within three hours after parturition. He agrees with the earlier statements of Aldrorandi, Legullois, Fraser and Schultz regarding the length of gestation, or period of pregnancy, as being about nine weeks, which is very nearly correct, sixty-two days being the normal length of time. He held that the return of the heat period did not follow any regular periodicity: "Wenn die Befruchtung unmittelbar nach der Geburt verhindert wird, so scheint die Wiederkehr der Brunst an keine ganz bestimmte zeit geknüpft zu sein, sondern von Umständen der Individualität, des Alters, der Jahreszeit, der Fütterung, etc., abzuhängen." In four cases in which the females were prevented from copulating for some time after they gave birth to young a copulation occurred 40, 50, 51 and 51 days after the birth.

Reichert ('61), confirmed the observations of Bischoff regarding the existence of a heat condition and an ovulation process shortly after parturition Reichert found many fertilized eggs in the oviducts 18, 19, 20 and 22 hours after parturition which showed by their condition that copulation must have taken place many hours before. His opinion is that the Graafian follicles rupture about twelve to fourteen hours after copulation.

Many recent authors have incorrectly stated Reichert's position and assert that he claimed ovulation in the guinea-pig not to be spontaneous but to depend upon copulation. This is due to a misinterpretation of Riechert's ideas, originated by Bischoff in his second paper, 1870, which is chiefly an answer to Reichert's arguments. No doubt many of the incorrect notions regarding Reichert's position have resulted from authors reading this paper by Bischoff without referring to Reichert's own paper for his exact position.

Reichert explains his position very clearly as follows:

> Es wäre wünschenswerth die Zeit genau angeben zu können, in welcher das Ei nach der Begattung aus dem Graaf'schen Follikel ausgestossen wird um die Einwirkung der Begattung auf das Austreten der Eichen bemessen zu können. Es ist zwar zu keiner Zeit auch nur wahrscheinlich gewesen, dass das bis zu den Eierstöcken vordringende Sperma irgend wie direkt die Lösung der Eichen oder richtiger das Bersten der Graaf'schen Follikel bewirken könne. Es ist ferner die bei anderen Thieren bekannte Tatsache, dass reife, selbst eingekapselte Eier auch ohne vorausgegangene Begattung gelöst werden durch Bischoff's Versuche *auch für die Säugethiere ausser Zweifel gesetzt.* Das Bersten aber der Graaf'schen Follikel erfolgt unter vermehrtem Zudrang des Blutes zu denselben und in Folge der starken Vergrösserung ihres Inhaltes, des gallertartigen Fluidums und auch der Zellen der Membrana granulosa, sowie des Discus proligerus; das Eichen selbst vergrössert sich in der Brunstzeit wenig oder vielleicht gar nicht; dasselbe löset sich nicht, es wird, so zu sagen, von der Mutter ausgestossen. Daraus geht ferner hervor, dass die Begattung mit ihren aufregenden Wirkungen auf das Mutterthier, insbesondere auf den Zudrang des Blutes nach den geschlechtstheilen, *einen sehr wesentlichen Antheil am Bersten des Graff'schen Follikels und so also an der Befreiung des Eichens haben kann und haben muss.*

This quotation shows that Reichert did not deny the existence of a spontaneous ovulation, but claimed that copulation had an important influence on the process of breaking the Graafian follicle. He also admits that the existence of a spontaneous ovulation is proven for mammals by the experiments of Bischoff. The difference between the opinions of Reichert and Bischoff is not that the one denies and the other admits the existence of a spontaneous ovulation, but that the one believes copulation to exert an important influence over ovulation, while the other holds that such an influence, if it exists at all, is not really great. Leo Loeb ('11), who has studied the problem of ovulation in the guinea-pig very recently, still claims that copulation exerts an influence over the time of ovulation. That Bischoff also finally thought that there might be an influence on ovulation as a result of copulation is shown by the following remark from his second paper:

> Sie meinen nur, es gäbe doch auch noch Erscheinungen, welche zeigen dass die Männchen und die Begattung auch einen Einfluss darauf ausüben. Wenn dieser Einwurf so gehalten wird, dass er (namely Reichert) zugesteht, die Erscheinung an und für sich ist vollkommen unabhängig von dem Männchen, dieses aber kann doch forderlich darauf einwirken, so wird dadurch nicht mehr gesagt, als wenn man sagen würde, eine gute Ernährung, günstige Verhältnisse der Temperatur und des Klimas haben ebenfalls einen Einfluss auf die Reifung und Loslösung der Eier, und diese vielleicht einen noch grosseren als die Gegenwart des Männchens und die Paarung. *Und wirklich steht auch gar Nichts entgegen, dem Männchen in diesem Sinne einen Einfluss einzuräumen.*

Hensen ('76), also recorded that in the guinea-pig a copulation takes place shortly (about one hour) after parturition and six to ten hours later an ovulation follows. In cases where this first ovulation was not followed by pregnancy he recorded another ovulation 17, 18, 35 and 37 days later in the different cases. The duration of pregnancy he found to be 66 days -This along with Bischoff's record of an ovulation 43 and 44 days after parturition made it difficult to admit that the guinea-pig had regular periodical ovulations every eighteenth day. Hensen, therefore, believed that the guinea-pig probably did not have a sharply expressed periodicity—"Es scheint also die Brunstzeit der Meerschweinchen nicht scharf periodisch zu sein."

Rein ('83), again reports the existence of a condition of heat in the guinea-pig within twenty-four hours after parturition.

Regarding the occurrence of further heat periods Rein failed to observe any regular periodicity. "Im Eintreten der Brunst habe Ich keine Periodicität bei den Versuchstieren bemerkt."

The foregoing studies are chiefly of historic interest yet they show that these earlier workers recognized the occurrence of ovulation shortly after parturition and were uncertain or confused regarding the time or periodicity of subsequent ovulations. Little of definite value has ever appeared in the literature to further clear up the last point. We may now briefly consider the more recent contributions which bear on the subjects of ovulation and oestrous in the guinea-pig.

Rubaschkin ('05), gives a detailed description of the sexual conditions in the guinea-pig. He also recognized, as did the earlier observers, that a condition of heat followed shortly after parturition. In almost all females killed a few hours (up to fifty hours) after the birth of a litter an ovulation had occurred. He never observed ovulation as early as five hours after parturition though he found fertilized eggs in the oviducts as early as fifteen and seventeen hours after. Copulation occurs directly after having given birth to young but for later heat periods Rubaschkin was unable to demonstrate any regular periodicity. "Es ist mir nicht gelungen, eine bestimmte Frist für das Auftreten der Brunst festzustellen."

He did observe, however, that in some animals ten to twelve days after having given birth to young the entrance of the vagina showed some signs of heat activity. "Oeffnung der Vagina und Röthung der Vaginalöffnung." He claimed that heat ceased to recur after the month of October, at least when the animals were kept in a cold place. The duration of pregnancy was reported by Rubaschkin in three cases to be ten weeks.

Rubaschkin thus failed to recognize the regular oestrous cycles in these animals and also states the gestation period somewhat too long.

Königstein in 1907 recorded the results of observations made on eighteen rats, one guinea-pig and five rabbits. He states that in the rodents heat occurs immediately after giving birth to the young and lasts for twenty-four hours. Copulation only takes place during heat and if pregnancy fails to occur at the period just after parturition the next heat periods follow after intervals of three to four weeks.

Königstein also examined sections of the genital tract giving some important histological descriptions based chiefly on the rat we shall return to a consideration of these observations in connection with our findings on the guinea-pig.

Bouin and Ancel ('10), are of the opinion that guinea-pigs do not have a spontaneous ovulation, the process being dependent upon copulation. However, these workers seem to have reached this opinion from observations made on rabbits which were the chief objects of their study. Despite the striking classification which they make of animals having a spontaneous ovulation (monkeys, dogs, horses, cows) and those not having spontaneous ovulation (rabbits, guinea-pigs, cats) they admit that rare exceptions are possible and that in any animal an ovulation might occur independently of a copulation.

> C'est là un fait général, mais soumis à des exceptions rares. Il peut arriver que des animaux à ovulation non spontanée opèrent la déchirure de leurs follicules mûrs en l'absence de tout rapprochement sexuel. Nous-même et M. Villemin avons constaté le fait chez le Lapin. M. Mulon vient également de l'observer chez le cobaye.

During the past several years Leo Loeb ('11 a, b) has contributed extensive and valuable studies bearing upon the sexual cycles in guinea-pigs, considering in particular the function and importance of the corpus luteum. Loeb examined a great number of ovaries at different periods, beginning with the time of the first copulation after parturition and concludes, as Rubaschkin 1905 and others had previously done, that the cyclic changes in the ovary take place independently of copulation. Loeb thought that the ovulations followed no exact and regular periodicity in all cases. The periodicity differed among the individuals and was influenced by certain external factors, particularly copulation. To quote:

> The exact time at which the new ovulation occurs varies however somewhat in different animals, ovulation occurring earlier in some ani-

mals than in others. In some cases it can be hastened through certain external factors, especially copulation, but in the large majority of cases it occurs sooner or later even without a preceding copulation.

He holds that eight days after ovulation large follicles are present in the ovary but sometimes ovulation may not occur for twenty or twenty-four days.

The 'sexual period,' period between two ovulations, according to Loeb lasts usually twenty to twenty-five days instead of being about two weeks, the time necessary for mature follicles to appear. This delay in ovulation in spite of the presence of mature follicles within eleven to thirteen days, he believes is due to a mechanism in the ovary which prolongs the cycle, the corpus luteum begins this mechanism. The corpus luteum degenerates after a period of growth lasting from seventeen to twenty days and thus ovulation occurs about once in three weeks. We shall show beyond by a demonstration of the oestrous cycles, that Loeb's deductions drawn from studies of the histology of the ovary are incorrect and, therefore, cannot be employed for determining the ovulation cycles in these animals.

Loeb further finds that when the corpus luteum is cut out immediately after an ovulation, the next ovulation occurs soon after mature follicles are developed—about thirteen to fifteen days. Under these conditions the normal sexual cycle is reestablished—but even here his periods are not exact being somewhat shorter than are actually normal.

The very varied time results obtained by Loeb may be given as follows: First, no ovulation has been found under normal conditions before the fifteenth day after the last copulation. Second, in a group of thirty-eight guinea-pigs killed fourteen days and eighteen hours and nineteen days and fifteen hours after the last copulation—one had ovulated about the sixteenth day, another the eighteenth and another at the nineteenth day, while the remaining thirty-five had not yet ovulated. Third, in a lot of twenty-two guinea-pigs, twenty to twenty-six days after the last copulation, one had supposedly ovulated at the eighteenth day, four at the nineteenth day, one at the nineteenth to twentieth day, one at the twenty-third and one at the twenty-fifth day and a half, while fourteen had not yet ovulated. Fourth, in a lot of six animals killed twenty-six to thirty-four days after the last heat period or copulation, only one had already ovulated.

A recapitulation of these results may be stated thus: under fifteen days no ovulation; sixteenth day, one; eighteenth day, two; nineteenth day, five; nineteenth to twentieth day, one; over fourteen days and eighteen hours and nineteen days and fifteen hours, thirty-five; twenty-third day, one; twenty-five and a half days, one; over twenty to twenty-six days, fourteen; twenty-sixth to thirty-fourth day, one; over twenty-six to thirty-four days, five. These figures as Loeb points out do not show any regularity in the occurrence of the ovulation process and, as we shall show beyond, they demonstrate how difficult or almost futile it is to attempt to solve the sexual cycles of an animal by a simple study of the ovarian conditions found on killing the animals at different periods. To anticipate slightly, the figures above show that Loeb entirely failed to discover the presence of a definitely regular periodicity in the ovulation process of the guinea-pig. Thus his examinations though much more thorough were as ineffective as those of the previous workers.

In 1913, Lams gave an instructive review of this problem. He again confirmed the long known fact that a heat period followed parturition in the guinea-pig. The copulation was found to take place within two to four hours after the delivery while ovulation occurred from twelve to seventeen hours after. Thus copulation generally preceded ovulation without being its cause. Lams gives no data on the occurrence of later ovulations but devotes himself to a detailed account of fertilization and the early development of the egg.

A consideration of the sum total of these various observations compels the admission that the opinions concerning the oestrous cycles in the guinea-pig are highly confused and totally unsatisfactory for application in exact breeding experiments. The one fact which presents itself was established by the earliest workers and confirmed by all subsequent studies—that is, that a period of heat follows within the first few hours after parturition. In the literature only Schulz ('29), according to Bischoff ('52), denies this fact.

No typical rhythm has been established so far for the subsequent ovulations in the guinea-pig. All observers who have examined a number of ovulations found great differences in the supposed periods of time intervening between two ovulations as we have reviewed in detail above. The numbers give no evidence of a regular periodicity in the ovulation process but on the contrary would lead one to believe that the greatest irregularity in time intervals was the rule.

On the other hand, really no observations exist to show anything like the occurrence of periodic changes in the uterus and vagina accompanying the return of the heat periods. Such a thing as a regular oestrous or preoostrous flow is completely undiscovered in these animals.

Königstein ('07), has examined sections of the uterus and vagina of a guinea-pig, and Blair-Bell ('08), has drawn comparisons giving many interesting observations, but they failed entirely, or made no attempt, to observe the regular reappearance of a definite order of changes in either the uterus or vagina of this animal.

3. OBSERVATIONS ON THE LIVING ANIMALS

During the past six years we have been using guinea-pigs in an extensive breeding experiment and it has become more and more evident as our work goes on that the existing notions of the ovulation periods in these animals are of no practical value, or are practically incorrect. In a number of the experiments it became important to know accurately when the females 'came into heat' and when ovulation took place. We had concluded, from numerous observations as well as theoretically, that the female guinea-pig very probably had a definitely regular and periodic sexual cycle if it could be worked out exactly. On account of the need of this exact information, we have studied the oestrous cycle in these animals during the past eighteen months.

Most other attempts at a solution of this problem have centered in a study of the ovary which necessitated either its removal by operation or the killing of the animal. In either case the procedure brought to a conclusion the observation or experiments on the ovulation cycles in that specimen. Recognizing, on the other hand, that no thorough investigation of the uterus and vagina in the living female had been made, it occurred to us that possibly oestrous changes might take place even though they are so feebly expressed as not to be noticeable on casual observation. The absence of an apparent oestrous or prooestrous flow from the vagina of the guinea-pig has, as before mentioned, no doubt been the chief reason for the general lack of knowledge of the oestrous cycle. It was therefore determined to make a minute examination of the contents of the vaginae of a number of females every day for a long peroid of time, to ascertain whether a feeble flow might exist although insufficient in quantity to be noticed at the vaginal orifice or vulva.

The observations were made by using a small nasal speculum which was introduced into the vagina and the arms opened apart by means of the thumb screw. The speculum permits an examination of the entire surface of the vaginal canal. In this way the vaginae of a number of virgin females have been examined daily and smears made from the substances that happened to be present in the lumen.

By the use of such a simple method, it was readily determined after examining the first lot of animals for a few months that a definite sexual period occurs lasting for about twenty-four hours and returning with a striking regularity every fifteen or sixteen days. During this twenty-four hour period the vagina contains an abundant fluid which is for about the first half of the time of a mucous consistency. The vaginal fluid then changes into a thick and cheese-like substance which finally becomes slowly liquified and serous. This thin fluid exists for a few hours and then disappears. Occasionally toward the end of the process a slight trace of blood may be present giving the fluid a bloody red appearance, otherwise it is milk-white or cream-color.

According to the changes in appearance and consistency of the vaginal fluid, one may distinguish four different stages. The first stage having a mucous secretion, a second stage the cheese-like secretion, a third stage with the fluid becoming serous and a fourth stage, not always recognized, during which a bloody discharge is present. The duration of these several stages is subject in the different animals to individual variations. The first stage, however, is generally longest and lasts from six to twelve hours or even more and during this time there is a gradually increasing quantity of the mucous secretion which at its height is very abundant and fills the entire lumen of the vagina. The second stage is shorter, lasting from two to four hours, and passes gradually over into the third stage which lasts from four to six hours. The fourth stage is the shortest, only about one to two hours long, and for this reason it is often missed in examining the animals during the periods. It is also possible, as mentioned above, that the fourth stage may not typically exist in all individuals and the quantity of blood present is very different in the different specimens. The succession in which these stages follow one another is remarkably definite. We have never observed any change in the typical sequence of the stages and the time consumed by the entire process is generally as stated about twenty-four hours.

A macroscopical examination of the uterus and vagina during this period of sexual activity shows the entire genital tract to be congested. The vessels to the ovary, uterus and vagina are large and conspicuous, the uterine horns and the vagina are slightly swollen and inflamed. However, as soon as this short period of activity is over, the congestion disappears and the uterus and vagina take again their normal pale aspect. At the same time the vaginal fluid diminishes and the vagina, especially during the first week after this sexual activity, is as clean as possible showing none of the secretion. The external vaginal orifice, which during the period of activity is more or less open actually showing in a few cases a little fluid or some blood, closes and becomes less accessible after the period.

During the second week following oestrus a little mucous discharge begins to appear in the vagina and increases progressively indicating that the new period of activity is nearer and nearer approaching. The orifice of the vagina is sometimes open during this stage and thus explains why this sign, which was observed before, does not make it possible to detect the actual time of the regular oestrous activity. Rubaschkin has observed the opening of the vagina ten to twelve days after parturition, but this period of time is certainly too short to indicate the return of heat. We agree with Rubaschkin in stating that during the ovulation the vagina is open, but we do not admit that the opposite is also true, that the opening of the vagina indicates unmistakably the return of the ovulation process.

4. MICROSCOPIC STRUCTURE AND CHANGES OF THE VAGINAL FLUID

A microscopical examination of the smears prepared from the vaginal fluid taken at the several stages separated above shows decidedly typical differences. The cellular character of a smear made at a given stage differs from the cellular make-up of all other stages. The relative numbers of various cell types in the fluid at different stages are so definite that one with a little experience may diagnose the exact sexual stage of the animal concerned solely by an examination of the smear.

A photomicrograph from a smear of the vaginal content during the first stage of mucous secretion is shown by figure 1. This mucous fluid is seen to contain an abundant mass of cells which, as shown in the figure, are of a squamous type with very small pycnotic nuclei sometimes broken into pieces. The cell protoplasm is also greatly degenerated having only a weak affinity for the plasma stains and exhibits a reticular structure. These cells derived from the wall of the vagina (fig. 17) characterize by their presence and great superiority in numbers this first stage. There are, however, to be seen particularly toward the end of the first stage a certain number of elongate, cornified cells without nuclei, which are desquamated from the more external portions of the vagina. These cells contrast in appearance with the first type cells since in smears stained with haematoxylin and eosin they present a decidedly red color, while the abundant first type cells are almost grey. The red cells rather serve to indicate an intermediate period between the first and second stages or periods of the flow, and may really be found during both stages but particularly at the end of the first and beginning of the second stage. In addition to these two kinds of cells other types may also be found in a first stage smear but they are never present in such abundance nor are they so typical as the two just mentioned. All of the cells float freely in the mucus without assuming any definite arrangement.

During the second stage the vaginal fluid is filled with enormous numbers of cells which cause the cheese-like consistency of the discharge at this time. These cells illustrated by the photomicrographs, figures 2, 3 and 4 at three different magnifications, are derived from the upper portions of the vagina with a few from the uterus and they maintain to a higher degree the original or healthy architecture of an epithelial cell. The nuclei are fairly well preserved showing only slight signs of degeneration. The protoplasm has not greatly deteriorated and gives a good staining reaction thus differing from the grey-staining first stage cells. The cells are present in innumerable quantities forming the thick cheesy substance while the mucous secretion diminishes more and more until it almost disappears. This stage is of short duration.

The third stage begins with the liquefaction of the cheesy mass. A microscopical examination shows that the cells of the second stage become less and less numerous, while a great number of polymorphonuclear leucocytes appear among them (figs. 5 and 6). When the end of this process is reached almost every one of the cells has become isolated from others of its kind and lies in the midst of a number of leucocytes. The apparent action or effect of the leucocytes is to dissolve or digest the desquamated epithelial cells and this dissolving effect is not only noticed on cells surrounded by the leucocytes but in some cases the leucocytes dissolve their way into the interior of the cell-bodies (figs. 7 and 8). These appearances are not due, as might possibly be supposed, to the cells having devoured the leucocytes. This destructive influence of the leucocytes begins, as will be described later, before the desquamated epithelial cells have fallen away from the wall of the uterus and vagina (figs. 15, 16 and 17). But it probably continues also after the cells are free in the lumen of the vagina. The dissolving power of the leucocytes, which probably causes the liquefaction of the cheesy mass of epithelial cells is shown very well when leucocytes are seen within a cell and the nucleus is beginning to dissolve. The nucleus is apparently digested and dissolved by coming in contact with the leucocyte without being at all engulfed or enclosed within the smaller body of the leucocyte.

As the third stage appoaches its end the material within the vagina is a thin fluid containing a great number of leucocytes as well as many epithelial cells of the second stage some of which contain leucocytes within their bodies. Such leucocyte containing cells are strikingly typical of the third stage. The leucocytes within these cells as would be expected very soon show signs of degeneration never staining so clearly as the free outside ones.

The fourth stage shows the same condition as the preceding but often at this time a slight hemorrhage takes place, though this does not always occur. A microscopical examination of the hemorrhagic fluid shows in addition to the great number of red blood corpuscles, a large number of leucocytes and also desquamated cells of the second stage, some of which are penetrated by leucocytes (fig. 9). Sometimes red blood corpuscles are enclosed within the bodies of the leucocytes and digested, this is probably a truly phagocytic action and not entirely the same as their dissolving effect on the neighboring epithelial cells within the fluid.

The presence of the leucocytes is not alone confined to the heat period but an abundant quantity of them is also to be found in the lumen of the vagina during the dioestrum. The only time that leucocytes are absent from the vaginal lumen is during the first and second stage described above at the beginning of the oestrus. Throughout the first week after heat the little fluid which exists in the vagina contains chiefly leucocytes and a few atypical desquamated cells. During the second week the number of epithelial cells increases more and more and among these atypical cells there may exist isolated cells of the first or of the second stage type.

At the fourteenth and fifteenth day the number of first stage cells already described begins to increase gradually and the growing proportion of these cells indicates the approaching new period of heat.

5. THE OESTROUS RHYTHM

The periodical return of a typical flow showing the above described macroscopical and microscopical details, was found to be very regular in twenty-six virgin females examined during different seasons of the year. Table I shows the results of this examination. As this table indicates, all the females examined were virgin thus eliminating any chance of modification which might be due to the act of copulation. Their ages ranged between three and a half and fifteen and a half months during the time of examination. The female guinea-pig is sexually mature at about three months old. Almost every animal, as the table shows, was examined for a length of time covering several oestrus periods. In the sixty-seven periods examined altogether the vaginal flow returned regularly every fifteen to seventeen days with an average of 15.73 days interval between the beginning of periods.

This table contains nine oestrus periods for operated animals from which one ovary was removed. The operation was done to determine whether any decided alteration in the oestrus would result after the loss of one ovary. The animals 1080 ♀ and 1102 ♀ were semi-spayed during the time of examination given in the table. In the animal 1080 ♀ the first heat period following operation came at the sixteenth day after the last period but in the animal 1102 ♀ the first heat period following operation came on the fourteenth day after the last heat, a little earlier than it should come inder normal conditions. The three heat periods following this came, however, very regularly every sixteenth day.

The animals 867 ♀, 923 ♀, and 1069 ♀ were semi-spayed a considerable time before the beginning of the examination and

Table I

RECORDS OF 26 GUINEA-PIGS EXAMINED DAILY FOR THEIR OESTROUS CONDITIONS

Animal	Condition	Examined from ages ✲	Dioestrous cycle in days	Total periods	Total days	Average days	Month examined									Remarks
							Jan	Feb	Mch	Apr	May	June	Oct	Nov	Dec	
746 ♀	Virgin	4-5½ months	15, 15	2	30	15					+	+				
753 ♀	"	4-5½ "	17, 16	2	33	165					+	+				
754 ♀	"	4-6 "	17, 16, 16	3	49	1633					+	+				
756 ♀	"	14-15½ "	16, 16, 16	3	48	16		+	+	+						
766 ♀	"	4-5½ "	16, 15	2	31	155					+	+				
768 ♀	"	4-5½ "	15, 17	2	32	16					+	+				
783 ♀	"	3½-5 "	15, 15	2	30	15					+	+				
786 ♀	"	3½-5½ "	16, 17, 15	3	48	16					+	+				
859 ♀	"	9-11 "	17, 16, 16, 17	4	66	165	+	+							+	
860 ♀	"	9-12 "	16, 16, 16, 16	4	64	16	+	+	+						+	
867 ♀	"	11-12 "	(17)	1	17	17			+							Only one ovary; right removed
923 ♀	"	11-12½ "	(17), (17)	2	17	17		+	+							Only one ovary, left removed
935 ♀	"	8-11½ "	16, 16, 16, 16, 15, 16	6	95	1582	+	+	+						+	
1011 ♀	"	7-11 "	16, 17, 16, 16, 16, 16, 16	7	113	1614	+	+	+	+					+	
1037 ♀	"	5-6 "	16	1	16	16								+		
1060 ♀	"	4½-5 "	16	1	16	16								+		
1066 ♀	"	4-4½ "	16	1	16	16								+		
1069 ♀	"	8-9 "	(17)	1	17	17		+	+							Only one ovary, right removed
1073 ♀	"	4-5 "	16, 16	2	32	16							+	+		
1074 ♀	"	4-5 "	16	1	16	16								+		
1080 ♀	"	5-7 "	16, 16, 16, [16]	4	64	16	+	+							+	Right ovary removed during examination period 2/1
1086 ♀	"	4-4½ "	16	1	16	16								+		
1089 ♀	"	4-5 "	16, 15	2	31	155								+	+	
1097 ♀	"	3½-4 "	15	1	15	15							+	+		
1102 ♀	"	5-8 "	16, 16, 16, [14], ⑯, ⑯, ⑯	7	110	1571	+	+	+						+	Left ovary removed during examination period 1/30
1104 ♀	"	4-5 "	16, 16	2	32	16								+	+	
Total				67	1054	1573	6	9	8	2	7	7	2	9	8	

✲ Guinea pigs are sexually mature at about 3 months old all of these showed an interval of seventeen days between the beginnings of the dioestrous cycles. The average of seven periods in animals with only one ovary is 16.57, this being much higher than the average of all the cases, which is 15.73 days. The average of the only two cases of first heat-periods after operation, on the other hand, is lower than the general average 15.0. The number of cases is, however, entirely insufficient to warrant a conclusion, though suggestive for further investigation. It is probable that when only one ovary exists, the period between ovulations is a little longer than under normal conditions. The two ovaries may alternate to a certain degree in their function or they may share the entire task in a less exhaustive way than one ovary is capable of doing. Semi-spayed females often have large litters which might indicate that the single ovary matured more follicles than would have been its share should the other ovary have been present.

Eliminating from the general table the results obtained by the examination of the semi-spayed animals, one finds an average of 15.65 days for the length of time from the beginning of one heat period to the beginning of the next in all normal cases. This we believe to be the length of the oestrous cycle of the guinea-pig under uniform conditions.

Table 1 further shows the months during which these observations were made. The animals were examined during early summer, fall, winter and spring and have shown at all seasons a perfect regularity in the return of the heat periods. Their oestrous cycle is certainly typically regular. The only months during which the animals were not examined are July, August and September. During the winter the guinea-pigs are kept in a fairly well regulated warm temperature running about 70° Fahrenheit on an average. It may be possible that in the wild state under natural conditions when the weather is cold and food somewhat scarce, the heat periods may cease for a season or become less frequent. Rubaschkin claimed that heat ceased to recur after October when guinea-pigs were kept in a cold place. But under the steadily favorable conditions in which the guinea-pigs here considered are kept, it is certain that they are sexually active throughout the entire year with an astonishingly regular return of their oestrous flow and breeding reactions.

A more careful consideration of the figures obtained during the different months indicates, however, that there probably is a small difference in the length of the sexual cycles during the warm and the cold seasons.

The curve shown in figure A indicates graphically this slight fluctuation, operated animals are excluded. The lowest average 15.50 days, or the shortest oestrous cycles, was found in the month of October, while the highest 16.14 days is shown during January. The heavy line at 15.82 days indicates the mean between these two extremes. It is probably not without significance that the averages during the months December, January, February, March and April fall above the mean line, while the averages during the months of May, June and October are below the line. From the cases considered this indicates that the length of the oestrous cycle is probably a little shorter during the warm time of the year and a little longer during the cold weather. We must, however, admit that the number of considered cases, as given in table 1, is actually small and these slight seasonal variations may be more suggestive than demonstrative in importance, yet there is certainly a striking consistency in their arrangement.

6. CYCLICAL CHANGES IN THE UTERUS AND VAGINA

After having determined the regularity of the dioestrous cycle in a number of virgin females, they were killed at different stages of the oestrous period and their ovaries as well as pieces of the uterus and vagina were carefully examined and then fixed and preserved for microscopical study. The uterus and vagina must be fixed in certain fluids to avoid shrinkage and a tearing away of the epithelium from the wall. Bouin's fixing fluid has proven most satisfactory for this purpose while the ovaries were generally fixed with Zenker's fluid.

During the dioestrum or resting period the uterus is lined by a layer of cuboidal ciliated epithelium. Figure 10 shows a sec-

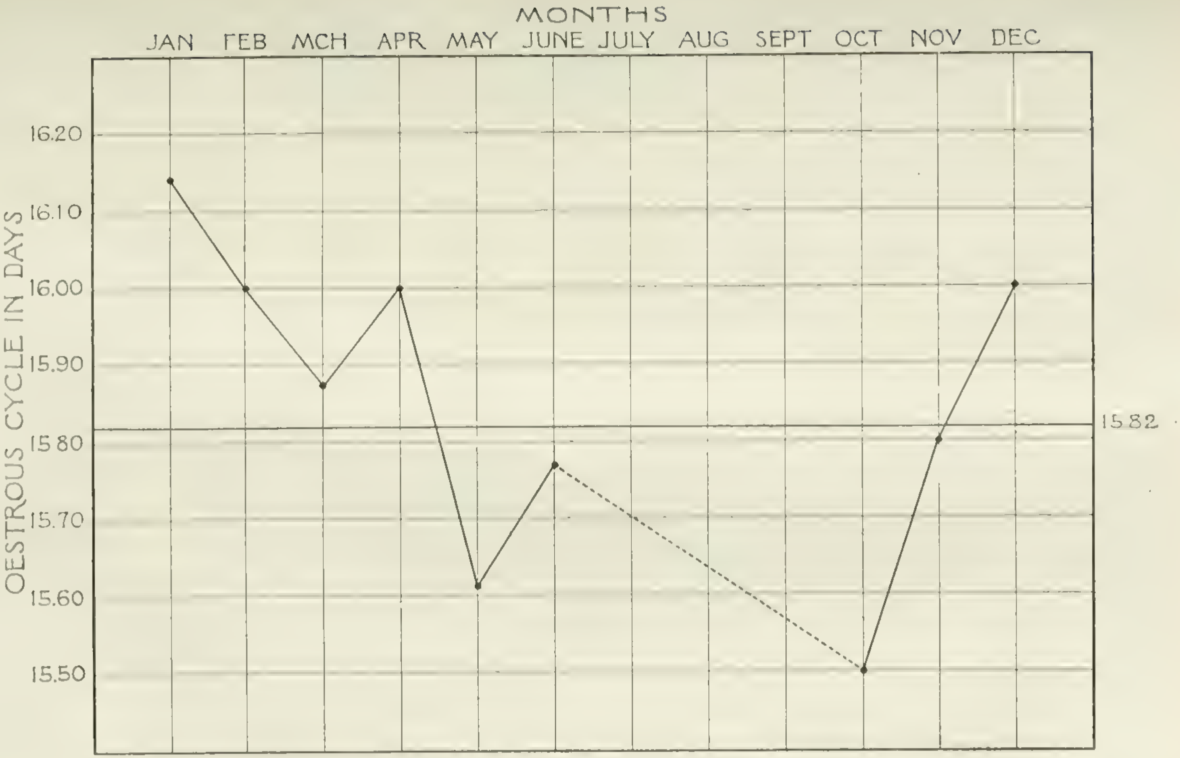


Fig. A Chart showing the average length in days of the guinea-pigs' dioestrous cycles during the different months of the year. The cycles are slightly longer during the colder months.

tion through the uterus at four and a half days after the last oestrus. At this time the epithelial cells present a normal and vigorous aspect. No loss or breaking down is to be noticed. A few leucocytes are occasionally seen among the cells of the stroma, but never in large numbers. Mitoses are not frequent at this time but they are to be seen now and then.

When the heat period begins, the epithelium loses its normal appearance (figs. 11, 12 and 13). The epithelial cells become tall and columnar and are filled with mucus which they begin to form in abundant quantity The nuclei of the columnar cells appear closely pressed one against the other and are pressed into different levels in the various cells so as to give an appearance of several rows of nuclei. The epithelium thus takes on a pseudo-stratified arrangement. At the same time, a large number of leucocytes begin to migrate from the capillaries through the stroma and towards the epithelium. The stroma itself is congested and possesses a more profuse circulation than usual.

These appearances are to be seen in animals killed during the first phase of their period, that is, when the vagina contains an abundant mucous fluid filled with desquamated epithelial cells. A smear of this fluid is illustraed in figure 1.

As soon as the second phase of the vaginal fluid appears (figs. 2, 3 and 4), the uterus shows another aspect. The leucocytes are accumulating in large numbers below the epithelium, forming in some places a perfect wreath of leucocytes under the epithelium or actually a separate layer of cells (fig. 14). The stroma shows a more advanced degree of congestion.

During the third stage, smears figures 5 and 6, the leucocytes penetrate more and more into the epithelium some of them making their way into the lumen of the uterus by passing between the epithelial cells. Other leucocytes actually enter the epithelial cells and penetrate into their interior (fig. 15). A stage more advanced in appearance corresponding to a late third stage though from the same animal as figure 15, is shown in figure 16, where the entire epithelium is almost completely disintegrated. A great number of leucocytes has already penetrated the epithelium the cell structure of which has become largely destroyed.

Large vacuoles are to be seen between the epithelial cells, and these are probably produced by the dissolving power of the leucocytes. Under the destroyed epithelium haematomata are to be seen in several places, produced by the congestion of the peripheral capillaries in the stroma. A leucocytosis somewhat similar to the above has been described by Heape, Königstein, Blair-Bell and others in the uteri of several mammals.

The vagina of the guinea-pig also shows analogous conditions as illustrated in figure 17.

The broken down epithelium remains until the regeneration process begins. The reparation starts from the necks of the uterine glands which have remained intact during the entire process of destruction. A few leucocytes are to be seen between the epithelial cells of the uterine glands but this small number apparently passes through the epithelium into the duct without injuring the epithelial cells. The stage of reparation corresponds to the fourth stage, that is, to the period when blood is sometimes seen in the vaginal fluid, see smear figure 9. This is not difficult to explain since regeneration and the falling off of the degenerated epithelium take place at the same time. Regeneration of the uterine epithelium before the oestrous flow had ceased has been reported in other mammals.

After examining a number of specimens, one may get the impression that the new epithelium growing out from the neck of the glands tends to push off the old degenerate epithelium, as it becomes detached from the wall of the uterus. Figures 18 and 19 show this condition where the new and the old epithelium are still existing in close proximity, the one growing out from the gland, the other breaking away from the wall of the uterus. In figure 19, this condition is more advanced and one sees the old epithelium partly detached from the wall of the uterus. Generally the epithelium falls off still connected with pieces of the stroma, which also seems to be destroyed to some extent during every heat period. These masses of epithelial cells are commonly found in the vaginal fluid. When the epithelium falls away the haematomata are uncovered and the blood contained in them passes into the lumen of the uterus. A similar bleeding may also occur into the lumen of the vagina.

The regeneration of the mucosa seems to take place very quickly. About six to ten hours after the above stage the new epithelium is already completely formed. The growth of the new and the falling off of the old epithelium seem to go hand in hand, so that no stage is to be found when the uterus is completely unlined by its epithelial layer. However, one may occasionally observe, during the above described fourth stage, limited naked regions from which the old epithelium has been detached before the new has formed.

The wall of the vagina undergoes somewhat the same destructive changes as the wall of the uterus except that the desquamation of the vaginal epithelium does not occur in cell clumps or groups at the end of the third stage. The vagina merely sheds its epithelial cells singly but in increasing numbers from the beginning of the heat period up to the third stage. The desquamation appears to proceed from near the entrance up into the inner portions of the vagina. The cells which appear during the first stage come from near the outer part of the vagina, while during the second stage the desquamated squamous cells are derived from the inner part of the vagina. This statement does not include the cornified cells from near the orifice, which are found as mentioned above, between the first and second stages. The vaginal epithelium is also invaded by the leucocytes. This migration is very vigorous during the third stage, about the same time as in the uterus. An innumerable mass of polymorphonuclear leucocytes migrate into the vaginal epithelium and actually enter its more superficial cells by penetrating into their cell bodies (fig. 17).

The beginning of the desquamation before the massive arrival of the leucocytes shows that the primary cause of the desquamation is not the presence of the leucocytes. But, on the contrary it is probably the presence of the altered and dying desquamated cells which induces the extensive migration of leucocytes to this epithelial surface. The large epithelial cells of the vagina photographed in figure 17 are the same cells which are to be observed in the vaginal fluid during the third stage, see smears figures 6, 7 and 8. A congestion of the capillaries of the mucosa also takes place in the vagina, and slight hemorrhages may occur as in the uterus, when the destruction of the stratified epithelium chances to reach down to the tunica propria.

The leucocytes are chiefly attracted to that portion of the epithelium covering the outfoldings into the lumen and this part undergoes a greater destruction. In a similar way it is the epithelium covering the prominent folds of the uterus which is destroyed, while the ingrowths which form the uterine glands are preserved and through regeneration from their necks furnish the new material which is necessary for the restoration of the lost epithelium.

During the dioestrum or rest period the desquamation of epithelium from the vagina does not stop completely and the scant vaginal fluid always contains some desquamated cells. At the same time, and probably connected with the shedding process the exodus of the leucocytes also continues though in a less active way than during heat. The 'intermenstrual fluid' therefore always contains a considerable number of leucocytes.

7. THE OVARIAN CYCLE

A study of the ovaries fixed during different stages of the oestrous cycle has shown that every change taking place in the uterus and the vagina has its corresponding stage of change in the ovary. At the beginning of the first stage the ovaries possess large, ripe follicles, figures 20 and 21. The nuclei of the eggs contained in the follicles are in a resting condition. The theca folliculi shows the beginning of a slight congestion. As the first stage advances this congestion becomes more and more pronounced and by the beginning of the second stage it is highly developed, figures 22 and 23. This extreme congestion of the theca folliculi, which exist at about the same time as the congestion stage in the uterus (cf. fig. 14) indicates that the follicle is ready for rupture. Heape has pointed out that the rupture of the follicle is due to this congestion and if the ovarian blood supply be tied off follicles do not rupture. During this time the nucleus of the egg is still in a resting condition.

The ripe follicles break at about the end of the second or the beginning of the third stage. Figure 24 shows a follicle just broken at the commencement of the third stage. It will be recalled that at this time the active leucocytosis begins in the uterus and the vagina, compare figures 15, 16 and 17. The ovaries are not omitted from this active migration of the leucocytes. A number of leucocytes are to be seen in the corpus luteum during its early development, but great numbers of leucocytes are to be found mainly in the atretic follicles, which are now becoming the seat of regressive and degenerative processes (fig. 25). The eggs in these disorganizing follicles show a peculiar activity expressed by the formation of the maturation spindle. Most of the eggs begin to degenerate before the formation of a polar body, though some of them succeed in completing their maturation divisions. Figure 25 shows an egg within a disintegrating follicle, the follicle containing a great number of leucocytes. This egg possesses a well formed polar body in the process of division. Kirkham has reported similar conditions in the ovary of the mouse, he notices that eggs degenerate after forming the first polar body and the second polar spindle, a condition closely similar to that shown in our figure 25. The outline of the polar body is clearly shown in the specimen. The photograph is not 'touched up.'

The chromatin of the nucleus is to be seen in the center of the egg in figure 25. In all the cases observed, the eggs of the atretic follicles degenerated, the nucleus breaking up into irregular pieces very soon after ovulation had taken place from the ruptured follicles. We failed to find anything to indicate a tendency toward parthenogenetic divisions in the many specimens which we have examined as Leo Loeb reported for these animals.

The ruptured follicles very quickly begin to undergo a reorganization resulting in the formation of the corpora lutea. Even during the third stage the corpus luteum is a well circumscribed body beginning its differentiation by the ingrowth of the vascular tissue of the theca folliculi into the hypertrophied follicular epithelium (fig. 26). This condition is more advanced during the fourth stage, when reparation begins in the uterus. Figures 27 and 28 illustrate two corpora lutea from the same ovary during the stage of uterine hemorrhage, the two are cut in different directions. The ingrowth of the vascular tissue toward the central cavity is apparent in these two figures. A well formed mature corpus luteum is shown in figure 29, taken from a section through the ovary of an animal about four and a half days after the heat period when the uterus was in a typical resting condition (fig. 10).

8. GENERAL CONSIDERATIONS

After a review of the above described facts there are several problems of general importance which may be profitably discussed in connection with them.

A fact of considerable significance is that the development and the degeneration of the uterine and vaginal mucosa corresponds very closely to the development and degeneration of the corpora lutea in the ovaries. At the time when the corpora lutea are highly developed and apparently active the mucosae of the uterus and vagina show a normally vigorous and healthy condition (cf. figs. 10 and 29). While on the other hand when the corpora lutea begin to degenerate during the second week after the 'heat period' the mucosae of the uterus and vagina also begin to show signs of degeneration and the process of desquamation slowly commences. At about two weeks after the last 'heat period,' when the wholesale destruction of the mucosa begins, the corpora lutea are almost completely degenerated.

The breaking of the Graafian follicles occurs during the oestrus as a result of a congestion which began in the theca folliculi at about the same time as the congestion of the stroma of the uterus and vagina. And finally when the regenerative growth of the uterine mucosa sets in, the ovaries then possess new corpora lutea in an active state of differentiation which were derived from these recently ruptured follicles.

These occurrences argue very decidedly against the theory advanced by Fraenkel ('03), and until recently supported by a number of other investigators. Fraenkel believed that the corpus luteum is the cause of the menstrual condition, producing through its secretion the destructive changes in the uterus and vagina. Such a supposition does not in any sense accord with the phenomena as they appear in the guinea-pig. If there is to be ascribed to the secretion of the corpus luteum an action upon the uterine and vaginal mucosae such an action is not of an injurious but of a protective nature. As we shall bring out further, the most plausible opinion of the action of the corpus luteum in the ovary itself, may also be interpreted as of a protective nature since it seems to prevent rupture of the Graafian follicles and the discharge of the ova. The facts obtained in the present investigation might not fully warrant the position that the corpus luteum really exerted an actively protective influence over the uterine mucosa, but they certainly in no sense suggest, and actually speak against, any injurious action on the mucosa by the secretion of the corpus luteum.

At the same time it is difficult to maintain that the absence of the protective action of the corpus luteum is the only or actual cause of the oestrous activity. The cause of oestrous is very probably more complex and the definitely regular rhythmical changes which take place in the uterus and vagina of the guinea-pig can not be fully explained as due alone to the degeneration of the corpus luteum. The absence of the luteal secretion possibly merely permits the uterine flow to occur as it seems also to permit the rupture of the ripe Graafian follicles. While the real mechanism determining the uterine reaction is a more complex factor and relatively independent, but affected in its expression by a close inter-relationship with the ovaries.

The various theories, however, which attempt to localize the cause of the uterine changes in the ovary are not in any case fully in accord with all the facts. It is of course true that the existence of the ovaries is necessary for the normal development and function of the uterus and vagina, and also that the removal of both ovaries leads to a disappearance of the typical oestrous changes in the uterus and finally to a degeneration of this organ. Yet the complete removal of the ovaries does not always prevent the menstrual periodicity from expressing itself in an atypicl but regular way for a considerable time afterwards (see Halban).

Our observations on three females from which both ovaries have been completely removed, show that such an operation does not fully abolish the return of the destructive menstrual changes as is generally claimed. But on the other hand, the absence of the ovaries promotes and prolongs the continuation of these destructive changes in such a way, that instead of a periodical menstruation, these spayed females have a long, continuous and atypical destruction of the uterine and vaginal mucosae, which leads finally to the degeneration of these organs. In some cases a distinct periodicity may be perceived, indicating that the rhythm of the menstrual activity may exist independently of the ovaries. The phenomenon that really is abolished and absent from the uterus after the removal of the ovaries is the return of any regenerative or reconstructive process which we believe is normally due to a secretion from the newly formed corpora lutea.

From such a view of these phenomena one may draw the following general conclusions: The oestrous changes in the uterus are regulated by two different factors, one direct and the other indirect. A secretion elaborated in the ovary apparently by the corpus luteum is necessary for the normal development and persistence of the uterine and vaginal mucosae. The absence of the secretion leads to regression and degeneration of the uterine tissue. Yet this control is not the entire explanation of menstruation. The regulation of this process and the return of definite changes in definite periods of time may possibly be due to the existence of a fixed mechanism somewhere outside the ovary. The rôle of the ovary and especially of the corpus luteum is not to produce but to permit and to stop the menstruation. Our conceptions correspond completely with the ideas of Halban, who has recognized the protective rôle of the ovaries upon the uterus and the vagina and the existence of a separate causal factor of menstruation independent of the ovary.

Fraenkel's theory that the corpus luteum is an active factor producing menstruation does not correspond with our observations. Neither, on the other hand, does the assertions of Marshall and Runciman that "the corpora lutea evidently exert no influence on the occurrence of heat" seem to us justified. Marshall and Runciman ('14), have advocated the importance of the interstitial cells in considering the ovarian factor concerned in the recurrence of the oestrous cycle as opposed to any active effect of the corpora lutea. They point out the evident incorrectness of the old views that the ovaries and uterus are related by a nervous connection. Transplantation experiments have shown the fallacy of such a notion and have demonstrated the presence of an internal secretion from the transplanted ovarian mass. Marshall then in arguing against the importance of the corpus luteum uses Heape's ('97), observations which showed that in monkeys menstruation might take place in the absence of either ripe follicles or newly formed corpora lutea. This observation, it seems to us, does not in any way point towards the interstitial cells as being important. Nor does it argue against our view that the absence of the corpora lutea permits menstruation and that their presence exerts a protective influence over the uterine mucosa. Heape's observation is perfectly in accord with this and it is to be expected that corpora lutea should be either degenerate or absent when menstruation occurs.

Marshall and Runciman performed operation experiments on four bitches. At these operations they attempted to destroy the large Graafian follicles by pricking with a knife or needle. In the first fox terrier at least nine follicles were injured in this manner. But one who has operated on the dog's ovaries knows how difficult it would be to discover all of the ripe follicles and almost impossible to get those on the dorsal surface of the ovary which is often closely bound down and almost covered. Yet it is not necessary in this discussion to question the destruction of every ripening follicle since the photomicrographs, which the authors publish, show that corpora lutea formed after the rupture of the follicles, and they state that the follicles artificially ruptured changed "into structures almost identical with normal corpora lutea"—except that development was not sufficient to fill the central cavity.

In the first two animals, which were their best experiments, since the time of the expected 'heat period' was fairly accurately known, the 'heat' came on about the time, or perhaps a little later, than it was expected and was not greatly influenced by the operation. This is just what we should expect on our supposition of the function of the corpora lutea. The dog is a monoestrous animal with a long anoestrous period and the destruction of Graafian follicles a few weeks before the oestrus was expected would have no bearing on the probable function of the corpora lutea in bringing on this period. The old corpora lutea resulting from the last ovulation were not disturbed and were probably just about degenerating and thus permitted the oestrus to occur very near the normal time. While the newly formed corpora lutea resulting from the operation were not sufficiently vigorous in their action to do more than slightly delay the menstruation.

Marshall and Runciman concluded that it is evident that the occurrence of 'heat' in the dog is not dependent upon corpora lutea, and that "The ovarian interstitial cells are possibly concerned in the process, but cyclical changes in the condition of these cells have not so far been observed in the dog's ovaries."

These conclusions and Marshall and Runciman's discussion are directed chiefly against Fraenkel's idea regarding the way in which the corpora lutea act; that is, the corpora lutea by their secretion perform an active function in bringing on the oestrous condition. We also disagree on the basis of the evidence furnished by the guinea-pigs with Fraenkel's views and for these animals at least such opinions are entirely incorrect. It seems to us, however, that Marshall and Runciman's experiments do not in any way argue against the position that the corpora lutea exert a protective influence over the uterine mucosa, nor that the absence or degeneration of the corpora lutea and the disappearance of its secretion permits the uterine mucosa to undergo the degenerative changes typical of the 'heat period.'

Therefore, we must object to their conclusion that the occurrence of heat is not dependent upon corpora lutea—and further we are unable to believe that their experiments, or any other so far recorded, indicate that "the ovarian interstitial cells are possibly concerned in the process." The evidence to our minds does not in the least point in such a direction.

A most ingenious attempt at an explanation of menstruation and one of the first logical views regarding the function of the corpus luteum was advanced twenty years ago by Beard in his monograph on the 'Span of gestation and the cause of birth.' According to Beard "Menstruation is comparable to an abortion prior to a new ovulation, and it is an abortion of a decidua prepared for an egg which was given off subsequent to the preceding menstrual period, and which had escaped fertilization."

In the earlier mammals, Beard imagines that gestation extended over only one ovulation period or short dioestrum of Heape's terminology. Thus prior to each ovulation, a birth would take place provided pregnancy had ensued after the previous ovulation, and if not the ovulation would be preceded by an abortive birth act. In this connection it is interesting to recall the well known fact that in man and other mammals abortions occur with a far greater frequency at the times for regular menstrual periods than at other times. In the human the time of the first menstruation after conception is a most critical period, and the time when the third menstruation should occur is responsible for the great predominance of three month foetuses to be seen in most collections, and so on up to the tenth period when the normal birth takes place.

In the evolution of mammals Beard calls attention to the tendency to develop a longer gestation period and more fully developed offspring, but in all cases the length of the gestation period is a multiple of the primitive ovulation periods. A reminiscence of the earlier primitive conditions still exist in all of the polyoestrous mammals. The gestation period of the guinea-pig extends over four oestrous cycles making it about sixty-two days long.

During pregnancy in higher forms, according to Beard's scheme the corpus luteum exerts a protective function by preventing a new ovulation and an abortive birth. In non-pregnant females, however, this abortive process is not counteracted by the quickly degenerating corpus luteum spurium and the uterus undergoes the changes of menstruation and a new ovulation occurs. This ingenuous theory aims to furnish an explanation of the periodically destructive changes occurring in the uterus and vagina of some mammals at the same time that the ovary is preparing to liberate its ova. And the chief virtue of the theory is that it points out the protective action of the ovary and especially of the corpora lutea on the uterine mucosa. Every menstruation process and every abortion reflex as well as every normal birth is the result of two different factors, one the condition produced by the absence of the luteal secretion and the other is the expression of a phylogenenetically and physiologically fixed rhythmical tendency within the uterus itself.

Beard's conception of the corpus luteum as an organ preventing ovulation has been adopted and further developed by many later investigators, Prenant, Sandes and Skrobansky, Leo Loeb, Ruge, Pearl and Surface, Halban and Köhler and others. All of these investigators have added evidence in favor of Beard's corpus luteum theory partly by new observations and partly by experiments on the living animals.

To state Beard's ('98, p. 101) position in his own words:

> The corpus luteum is probably a contrivance for the supression or rendering abortive of ovulation during gestation. The commencing degeneration of this structure some little time before the end of the gestation (like its rapid atrophy where fertilization has not taken place) allows of preparation being made for a new ovulation.

We are indebted to Leo Loeb ('11), for first putting these conceptions of Beard to experimental test. And Loeb showed that pregnancy as such does not prevent ovulation if corpora lutea are extirpated from the ovaries. Loeb also destroyed the corpora lutea in non-pregnant guinea-pigs and later examined the ovaries after different periods of time. In forty-two females the corpora lutea were destroyed by cutting them out completely with the following results: In one case the next ovulation had already occurred at the twelfth to thirteenth day (by the next ovulation is meant the ovulation following the last copulation) in one case at the thirteenth day, in five cases at the thirteenth to fourteenth day, in twelve cases at the fourteenth to fifteenth day, in four cases at the fifteenth to sixteenth day, in one case at the sixteenth to sixteenth day and a half, in one case at the sixteenth to seventeenth day, in one case after eighteen days, while in eight cases ovulation had not yet occurred at the time when the animals were killed.

Loeb also cauterized the corpora lutea in the ovaries of thirty-one guinea-pigs but the results, owing to the inferiority of this method, were not so satisfactory. The ovulation in some cases came at the fourteenth to fifteenth day, in other cases later. Loeb interpreted these experiments to indicate that the removal of the corpus luteum hastened the next ovulation. Such a conclusion is in no way actually contradicted by our observations, yet the experiments of Loeb are not completely satisfactory in the light of the present findings Loeb thought the usual sexual period, or time between two ovulations, in the guinea-pig was very much longer, and much more variable than it actually is. On such a basis it seemed that the ovulation period in the animals he examined had been considerably reduced. But as the present study shows the normal oestrous cycle in the guinea-pig is from fifteen to seventeen days, usually about sixteen days with very insignificant variations. So that the periods recorded by Loeb, after the operations are actually just about of normal duration. He found the greatest number of cases to ovulate after a period of fourteen to fifteen days (12 such cases or 28.57 per cent) and considered this much shorter than the normal condition, where as a matter of fact such a period differs only insignificantly from what we find to be the regular length of the oestrous cycle.

When we also take into account his method of calculating the days between the last copulation and the next ovulation, and especially the fact that he figured the ovulation time by the condition and probable age of the newly formed corpora lutea found in the ovaries examined, the slight variations are all very probably within the limits of error. We also believe that Loeb has been misled by the application of similar methods in calculating the normal sexual periods in these animals.

In order to test the influence of the removal of the corpora lutea on the following ovulation time, one must first definitely establish a normal ovulation period. Since this was not done we are forced to acknowledge that Loeb's experiments do not demonstrate the importance of the corpus luteum in regulating the ovulation process, though he must be credited for having definitely attacked the problem experimentally. Some doubt will also exist in the minds of those who have attempted the operation as to whether all of the corpora lutea are often to be removed from the ovary while it is in position in the abdomen.

We are not at all opposed to admitting the probability that the removal of the corpus luteum may shorten the usual sexual cycle. In fact such a discovery would accord with our notions of the function of the corpus luteum. We feel further that the present study has established the existence of a definite normal oestrous cycle and this knowledge makes the experimental analysis of the influence of the corpus luteum much more readily approached.

The knowledge of a typical and regular sexual cycle in the guinea-pigs as here demonstrated, paves the way for a better and more uniform understanding of the oestrous conditions prevailing in the different classes of mammals. All cases that have been studied with sufficient care give evidence at least of some rhythmical activity. The absence of external signs of oestrus in a great number of mammals, one of which was the guinea-pig, is the most evident cause of a lack of understanding of their sexual periodicity. It is to be hoped that the application of the simple method of examination of the vaginal fluid used in the present study may enable workers to readily obtain a clearer understanding of the sexual activities of other commonly used laboratory animals as well as mammals in general, since such information is of the greatest value in all exact experimental breeding.

The typical oestrous cycles are probably more regularly expressed among mammals living in a state of domestication, and consequently under steady environmental conditions, than among their relatives living in the wild, where the existence of great disturbing factors, especially variations in food and temperature conditions, may tend to modify their behavior. The evidence of such modification by these disturbing factors is the existence in most mammals of differences in their sexual behavior during the different seasons of the year. Such seasonal variations are frequently lost under uniform conditions of temperature and feeding as is the case with rabbits, and also with guinea-pigs if these show seasonal changes in their native wild.

It has been reported by some investigators, Rubaschkin and others, that guinea-pigs in captivity breed less frequently in winter than during the warmer months, though they may become pregnant at any season. Such results are probably due to a failure to keep the animals properly warm during winter.

Guinea-pigs under the uniform conditions of our experiments do not show any apparent changes in their sexual rhythm with the seasons, but as indicated on previous pages, it is probable that their sexual cycle is a little shorter during the summer than in winter, yet even this difference does not seem to be very definitely expressed.

9. SUMMARY

The above description of the details of the oestrous cycle in the guinea-pig may be briefly summarized as follows:

1. Guinea-pigs kept in a state of domestication and under steady environmental conditions possess a regular dioestrous cycle repeating itself in non-pregnant females about every sixteen days throughout the entire year with probably small and insignificant variations during the different seasons.

2. During each cycle typically corresponding changes are occurring in the vagina, the uterus, and the ovary; a given stage in one of these organs closely accompanying parallel stages in the other two.

3. Each period of sexual activity lasts about twenty-four hours and is characterized by the presence of a definite vaginal

fluid, which is not sufficiently abundant to be readily detected on the vulva but is easily observed by an examination of the interior of the vagina.

4. The composition of the vaginal fluid changes with the several stages of change occurring in the uterus and vagina.

a. To begin with, during what we term the first stage, the fluid consists of an abundant mucous secretion containing great numbers of desquamated vaginal epithelial cells. At this time sections of the vagina show an active shedding or desquamation of its epithelial lining cells. The cells of the uterine epithelium are loaded with mucus, and an active migration of polynuclear leucocytes is taking place from the vessels of the vagina and uterus out into the stroma and towards the epithelial layer.

b. During the second stage the contents of the vagina become thick and cheese-like on account of the great accumulation of desquamated epithelial cells. The walls of the uterus and vagina become congested and the migration of leucocytes becomes still more active.

c. The leucocytes reach the epithelium and vigorously invade its cells and intercellular spaces during the third stage. These wandering cells become enclosed within and apparently dissolve the breaking-down dead cells of the epithelium. The vaginal fluid becomes thinner under the dissolving or digesting action of the leucocytes. The congestion in the uterus and vagina becomes still more pronounced giving rise to small blood masses or haematomata beneath the epithelium. The epithelium of the uterus is highly disorganized, vacuolized and richly invaded by the leucocytes, so that portions of it fall away en masse actually carrying with it in some cases cells of the stroma.

d. The fourth stage is merely a continuation or result of the activities of the third. The falling away of the epithelial pieces and stroma cells permits the escape of the small haematomata or blood knots thus causing a slight bleeding into the lumen of the uterus and vagina. These traces of blood often give a redish aspect to the vaginal fluid. At this same stage a regeneration process begins from the necks of the uterine glands and also apparently from the epithelial infoldings in the vagina, so that the lost epithelium becomes rapidly replaced almost before it has ceased falling away. If one may picture the epithelial surface of the uterus and vagina as consisting of innumerable prominences and depressions, it may be said that the destructive processes mentioned above are largely confined to the epithelium covering the prominences and that this epithelium is finally restored by regeneration from the epithelium lining the depressions, or in the case of the uterus from the epithelium of the uterine glands. The congestion with the diapedesis of corpuscles and the formation of the blood haematomata and the great accumulation of leucocytes all occur chiefly in the outpushed or protruding parts of the uterine wall.

The regeneration process in the guinea-pig is very short, lasting only a few hours, from six to twelve in all.

5. Ovulation seems to occur spontaneously during every heat period without exception. The rupture of the follicles with the consequent ovulation takes place about the end of the second stage or the beginning of the third; that is, during the presence of the thick cheese-like vaginal fluid.

6. During the dioestrum or intermenstrual period there is very little fluid to be found in the vagina. This scant fluid consists of mucus in which are some atypical squamous cells from the vaginal wall and many leucocytes. A number of the leucocytes are old but there are probably new ones arriving almost continuously from the wall of the vagina. The only time at which the vagina seems to be practically free of leucocytes is immediately before and during the first and second stages of the oestrous period described above.

7. A marked correlation exists between the oestrous changes in the uterus and the developmental cycle of the corpora lutea. When the corpora lutea are highly developed and apparently active the mucosae of the uterus and vagina show a normally vigorous and healthy condition. While, on the other hand, when the corpora lutea begin to degenerate during the second week after the 'heat period' the mucosae of the uterus and vagina also begin to show signs of degeneration and the process of desquamation slowly commences. At about two weeks after

the last 'heat period,' when the wholesale destruction of the mucosa begins, the corpora lutea are almost completely degenerated. The breaking of the Graafian follicles occurs during the oestrus as a result of a congestion which began in the theca folliculi at about the same time as the congestion of the stroma of the uterus and vagina. And finally when the regenerative growth of the uterine mucosa sets in, the ovaries then possess new corpora lutea, in an active state of differentiation, which were derived from the recently ruptured follicles.

It, therefore, might be imagined that the secretion from the corpora lutea exerts a protective influence over the uterus and vagina while the absence of this secretion permits the breaking down and degeneration of the uterine epithelium typical of the 'heat period.'

10. LITERATURE CITED

Beard, J. 1897 The span of gestation and the cause of birth. A study of the critical period and its effects in mammals. Fischer, Jena.

1898 The rhythm of reproduction in mammalia. Anat. Anz., vol. 14.

Bischoff, Th. L. W. 1844 Beweis der von der Begattung unabhängigen periodischen Reifung und Loslösung der Eier der Säugethiere und des Menschen. Giessen.

1852 Entwickelungsgeschichte des Meerschweinchens. Giessen.

1870 Neue Beobachtungen zur Entwickelungsgeschichte des Meerschweinchens. Abh. der Königl. Bayer. Akad. der Wissensch., vol. 10.

Blair-Bell, W. 1908 Menstruation and its relationship to the calcium metabolism. Proc. Roy. Soc. of Med., Obstet. and Gync. Sec., July, p. 291.

Bouin, P. et Ancel, P. 1909 Sur les homologies et la signification des glandes à sécrétion interne de l'ovaire. C. R. Soc. Le Biol., vol. 67.

1910 Recherches sur les fonctions du corps jaune gestatif. Jour. de Physl. et de path. génér.

Corner, G. W., and Amsbaugh, A. E. 1917 Oestrus and ovulation in swine. Anat. Rec., vol. 12.

Fraenkel, L. 1903 Die Function des Corpus luteum. Arch. für Gynaekol., vol. 68.

Halban, J. 1911 Zur Lehre von der Menstruation—Protective Wirkung der Keimdrüsen auf Brunst und Menstruation. Zentralbl. f. Gynaekol., vol. 35.

Halban, J., und Köhler, R. 1914 Die Beziechungen zwischen Corpus luteum und Menstruation. Arch. of Gynäkol., vol. 103.

Heape, W. 1899 The menstruation and ovulation of monkeys and the human female. Trans. Obstet. Soc., vol. 40.

1900 The sexual season. Quart. Jour. Mic. Sc., vol. 44.

1905 Ovulation and degeneration of ova in rabbits. Proc. Roy. Soc. London, vol. 76 B.

Hensen, V. 1876 Beobachtungen uber die Befruchtung und Entwickelung des Kaninchens und Meerschweinchens. Zeit. f. Anat. u. Entwick, vol. 1.

Kirkham, W. B. 1910 Ovulation in mammals with special reference to the mouse and rat. Biol. Bull., vol. 18.

Königstein, H. 1907 Die Veränderungen der Genitalsschleimhaut während der Gravidität und Brunst bei einigen Nagern. Arch. f. Physiol., vol. 119.

Lams, H. 1913 Ètude de l'oeuf de Cobaye aux premiers stades de l'embryogenèse. Arch. de Biol., vol. 28.

Loeb, L. 1911 a Über die Bedeutung des Corpus luteum für die Periodizität des sexuellen Zyklus beim weiblichen Säugetierorganismus. Deutsche Mediz. Wochensch. No. 1.

1911 b The cyclic changes in the ovary of the guinea-pig. Jour. Morph., vol. 22.

1911 c The cyclic changes in the mammalian ovary. Proc. Am. Phil. Soc., vol. 50.

Longley, W. H. 1911 The maturation of the egg and ovulation in the domestic cat. Am. Jour. Anat., vol. 12.

Marshall, F. H. A. 1903 The oestrous cycle and the formation of the corpus luteum in the sheep. Phil. Trans. B., vol. 196.

1904 The oestrous cycle in the common ferret. Quart. Jour. Mic. Sc., vol. 48.

1910 The physiology of reproduction. London, 1910.

Marshall, F. H. A., and Jolly, W. A. 1905 The oestrous cycle in the dog. Phil. Trans. B., vol. 198.

Marshall, F. H. A., and Runciman, J. G. 1914 On the ovarian factor concerned in the recurrence of the oestrous cycle. Jour. Physiol., vol. 49.

Pearl, R., and Surface, F. M. 1914 On the effect of Corpus luteum substance upon ovulation in the fowl. Jour. Biol. Chem., vol. 19.

Prenant, A. 1898 De la valeur morphologigue du corps jaune, son action physiologique et therapeutique possible. Rév. génér. d. Sciences pur. et appl., vol. 9.

Reichert, C. B. 1861 Beiträge zur Entwicklungsgeschichte des Meerschweinchens. Abh. d. Kgl. Preuss. Akad. d. Wiessensch, Berlin.

Rein, 1883 Beiträge zur Kenntnis der Reifungserscheinungen und Befruchtungsvorgänge am Säugetierei. Arch. f. Mikr. Anat., vol. 22.

Rubaschkin, W. 1905 Über die Reifungs—und Befruchtungsprocesse des Meerschweincheneies. Anat. Hefte., vol. 29.

Ruge, C. 1913 Ueber Ovulation, Corpus luteum und Menstruation. Arch. f. Gynäkol., vol. 100.

Sandes and Skrobansky (Quoted from Oppenheim's Handbuch der Biochemie III, I, p. 377).

Schulz, 1829 Observationes de cobayae. Hist. Nat. Diss. Berlin (quoted from Bischoff).

Stockard, C. R. 1912 An experimental study of racial degeneration in mammals treated with alcohol. Arch. Internal Med., vol. 10.

1913 The effect on the offspring of intoxicating the male parent and the transmission of the defects to subsequent generations. Am. Nat., vol. 47.

Stockard, C. R., and Papanicolaou, G. N. 1916 A further analysis of the hereditary transmission of degeneracy and deformities by the descendants of alcoholized mammals. Am. Nat., vol. 50.

PLATE 1

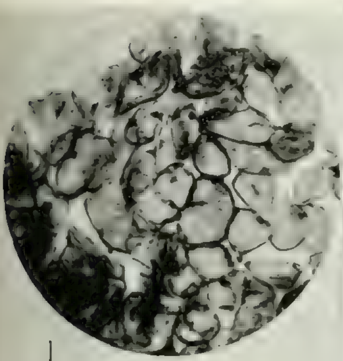
EXPLANATION OF FIGURES

The figures in all of the plates are photomicrographs made by Mr. Wm. Dunn of the Photographic Department of Cornell Medical School.

1 Squamous epithelial cells contained in the vaginal fluid during the first stage of oestrus from animal 1089 ♀. The vaginal fluid at this time is mucus filled with abundant cells of this type.

2 Cells from the second stage vaginal fluid. The great majority are squamous epithelial cells from the wall of the vagina with a few uterine epithelial cells. From animal 1066 ♀.

3 and 4 Cells of the second stage more highly magnified from 1066 ♀.



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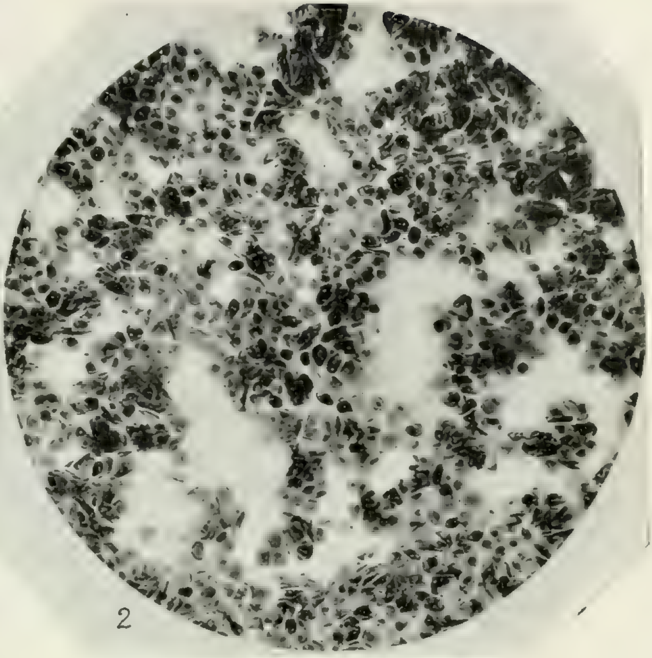

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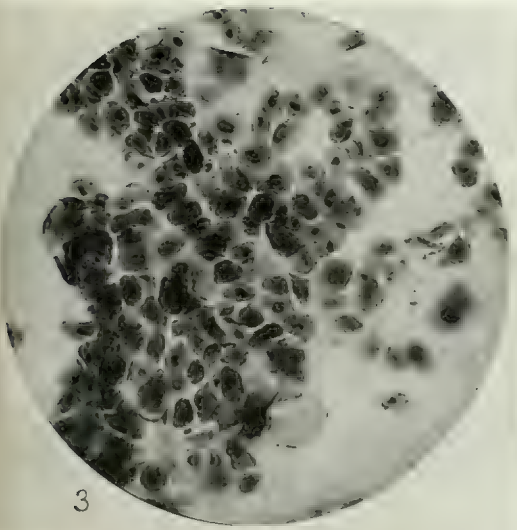

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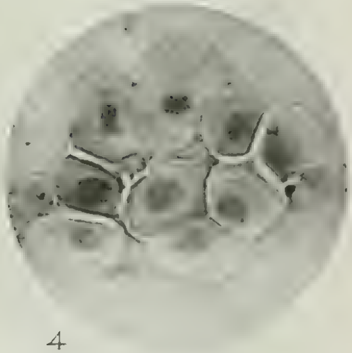

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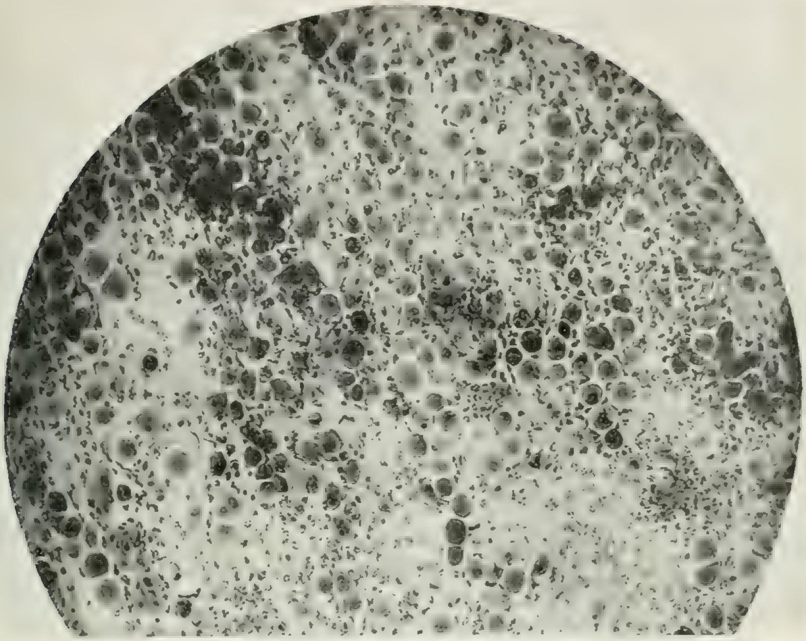
PLATE 2

EXPLANATION OF FIGURES

5 A smear of the fluid during the third stage, from animal 1104 ♀. This shows the arrival of myriads of leucocytes among the epithelial cells in the vaginal fluid. Such an appearance is characteristic of the third stage.

6 A more highly magnified view of the same stage showing in clearer detail the cell structures.



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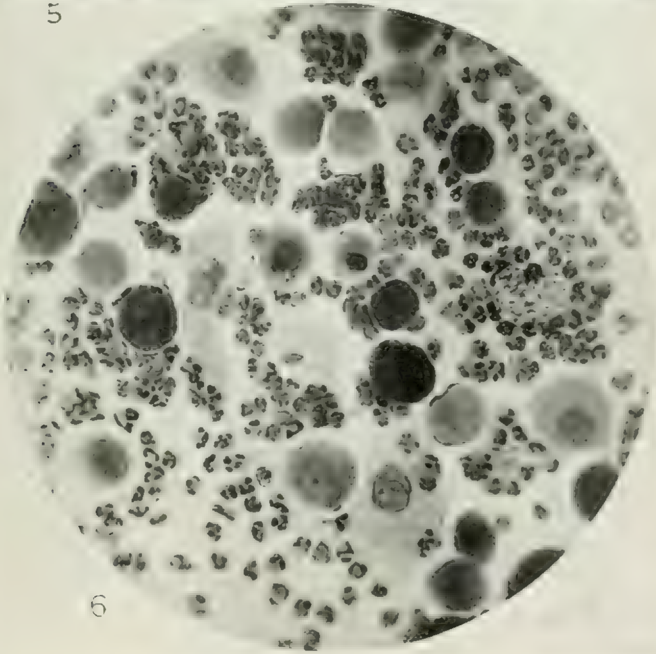

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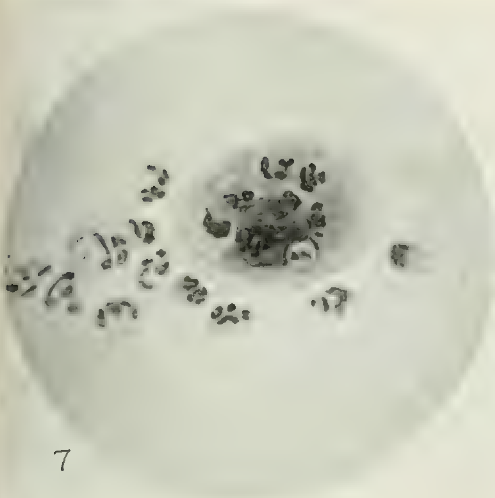
PLATE 3

EXPLANATION OF FIGURES

7 and 8 Highly magnified epithelial cells containing many leucocytes within their cell-bodies. A condition typical of the third stage—also from 1104 ♀.

9 A smear showing the presence of red blood corpuscles in the vaginal fluid during the short period of hemorrhage, following the third stage. From animal 1099 ♀. *rc*, red corpuscles.



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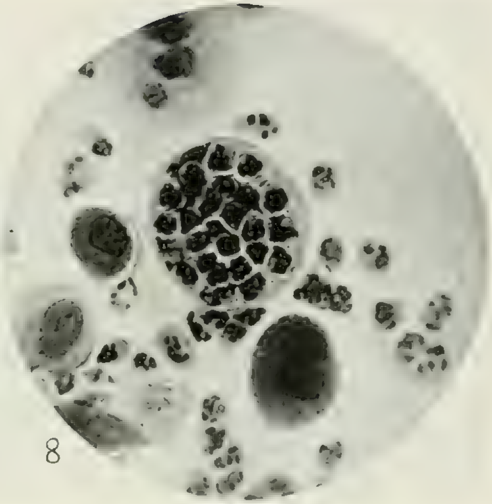

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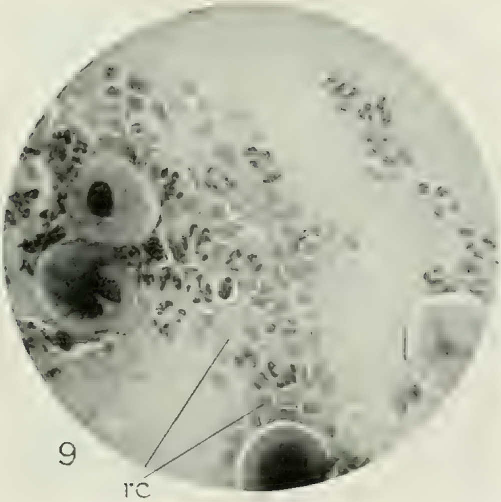

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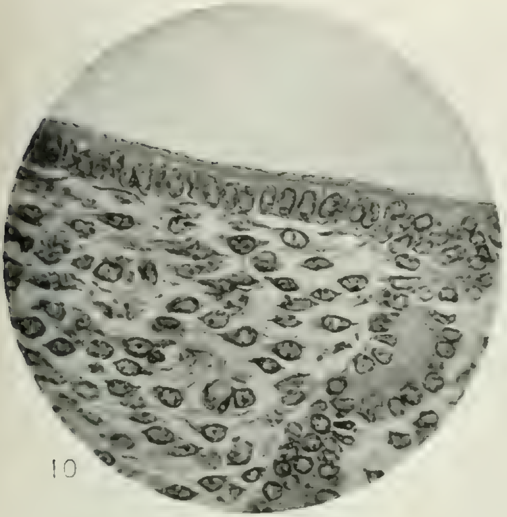
PLATE 4

EXPLANATION OF FIGURES

10 A section of the resting uterus during dioestrum, four and one-half days after oestrus, showing the normal cuboidal ciliated epithelium—animal 1074 ♀.

11, 12 and 13 Sections showing the condition of the uterine epithelium during its active secretion of mucus and the beginning of the leucocyte migration, from animal 1080 ♀ in which the oestrus was just commencing—*leu*, leucocytes. Note the contrast with figure 10. A corresponding smear of the vaginal fluid from the same animal just before it was killed is shown by figure 1.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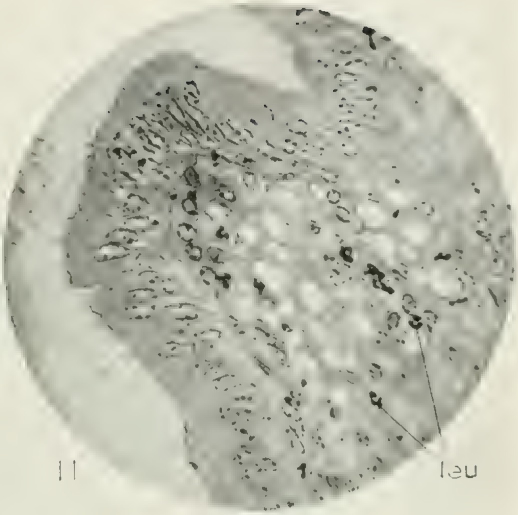
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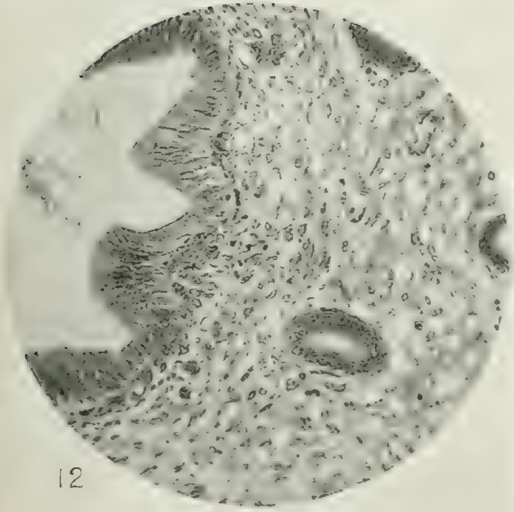
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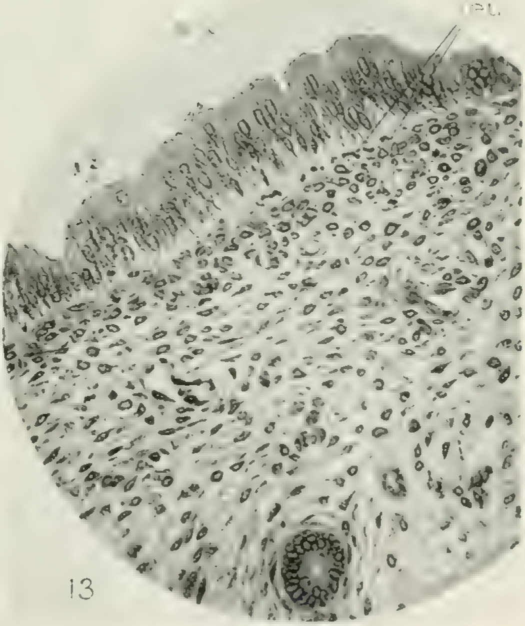
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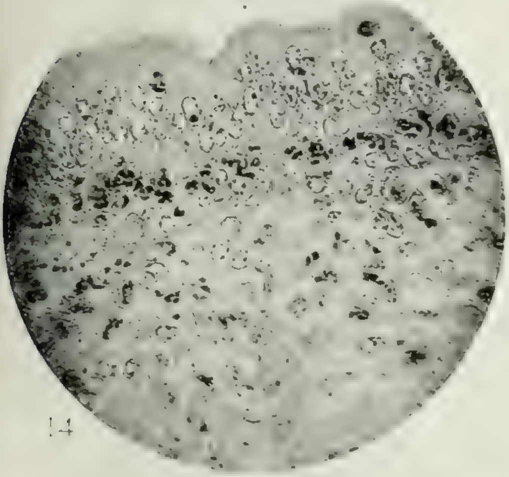
PLATE 5

EXPLANATION OF FIGURES

14 A section illustrating the condition of the uterine epithelium and the accumulation of large numbers of leucocytes below the epithelium during the second stage of oestrus, from animal 1066 ♀. Corresponding smears of the vaginal fluid at this time are shown in figures 2, 3, and 4 from the same female.

15 and 16 Sections of the uterus during the third stage of oestrus showing the invasion of the epithelium by migrating leucocytes. The epithelium is partially destroyed and greatly vacuolized, as a result of the dissolving action of the leucocytes, but is still adherent to the underlying stroma which also contains leucocytes. *leu*, leucocytes. Both sections are from 1104 ♀ and corresponding smears of the vaginal fluid from this animal immediately before being killed are shown in figures 5, 6, 7 and 8.

17 A section of the wall of the vagina from the same animal, 1104 ♀, during, of course, the same stage. The vaginal mucosa is also invaded by leucocytes in a manner similar to that of the uterus, several epithelial cells are seen to contain leucocytes within their bodies. The epithelium here is being desquamated or thrown off while the uterine epithelium is seen to be disintegrating before being shed. *leu*, leucocytes.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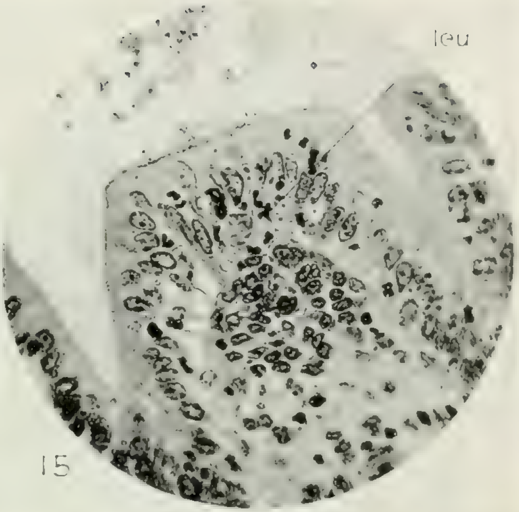

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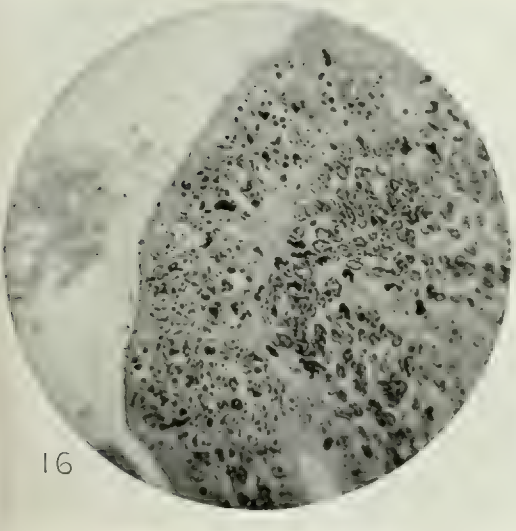
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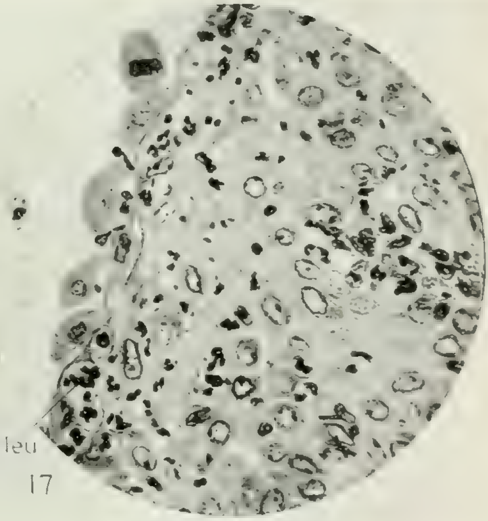

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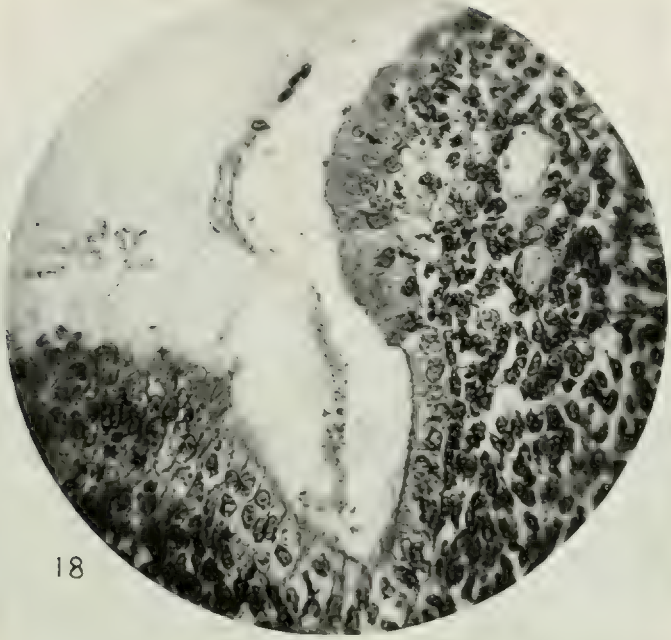
PLATE 6

EXPLANATION OF FIGURES

18 A section of the uterus from animal 1099 ♀ during the fourth stage, the short period of slight hemorrhage. The beginning regeneration of new epithelium from the neck of a uterine gland is shown while simultaneously the breaking down of the old epithelium is still taking place, and other portions of this section show a loss of the old epithelium from the uterine wall. A smear of the vaginal fluid from the same animal just before killing is shown in figure 9.

19 A similar section from the uterus of another animal, 860 ♀, during the same stage. This shows better the falling off of the old epithelium and the simultaneous formation of new epithelium.



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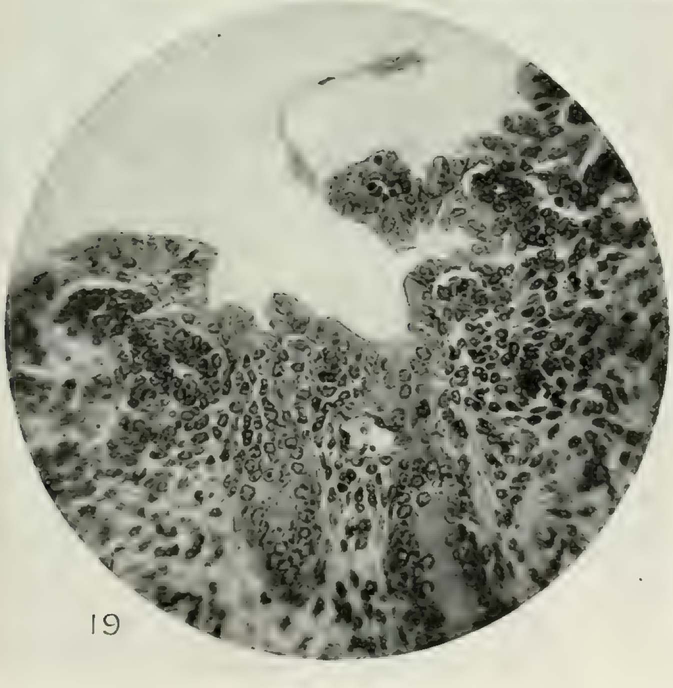

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PLATE 7

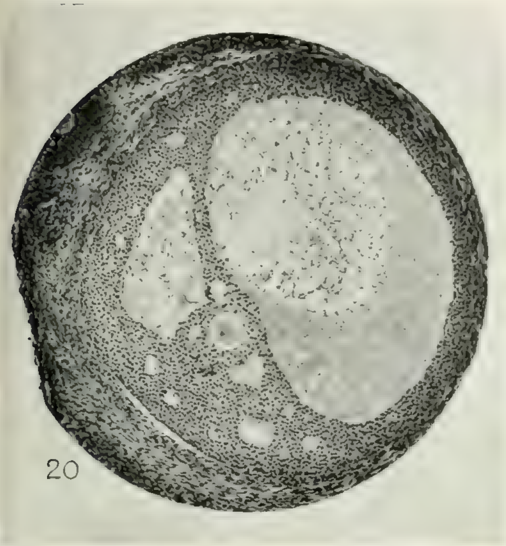
EXPLANATION OF FIGURES

20 A section of ovary from animal 1089 ♀ killed during the first stage of oestrus. A ripe follicle is shown a few hours before congestion of the theca begins. A smear of the vaginal fluid from the same animal is seen in figure 1 and sections of the uterus in figures 11, 12 and 13.

21 A higher magnification of the ovum and follicular wall shown in figure 20.

22 A section of the ovary from 1066 ♀ killed during the second stage of oestrus. The theca folliculi surrounding the ripe follicle has become highly congested. *bv*, blood vessels.

23 Shows at a higher magnification a clearer view of the congested condition of the follicle in figure 22, *bv*, blood vessels. The nucleus of the ovum is in a resting condition. Corresponding vaginal smears from this animal 1066 ♀ just before being killed are illustrated in figures 2, 3 and 4, and a section through the uterus in figure 14. All of these figures illustrate commonly seen second stage conditions.



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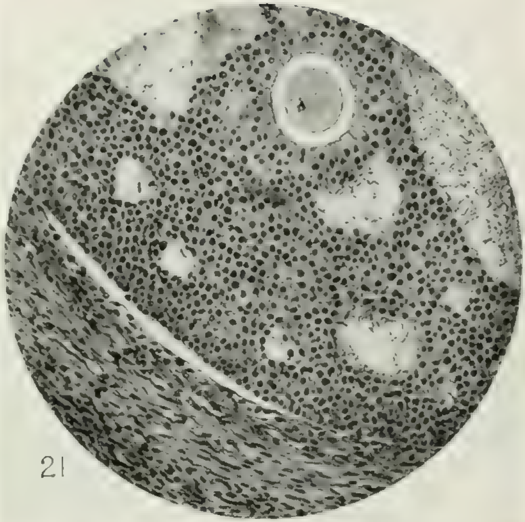

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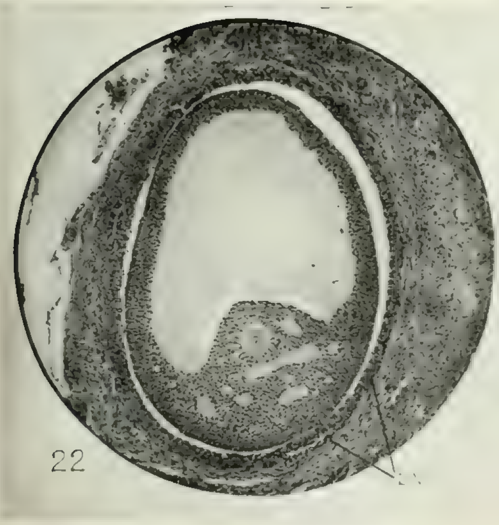

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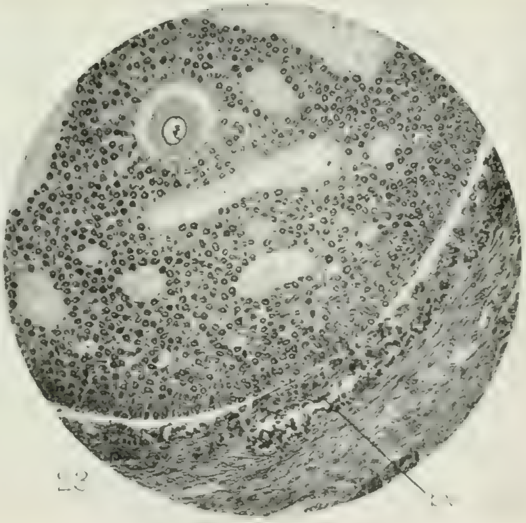

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PLATE 8

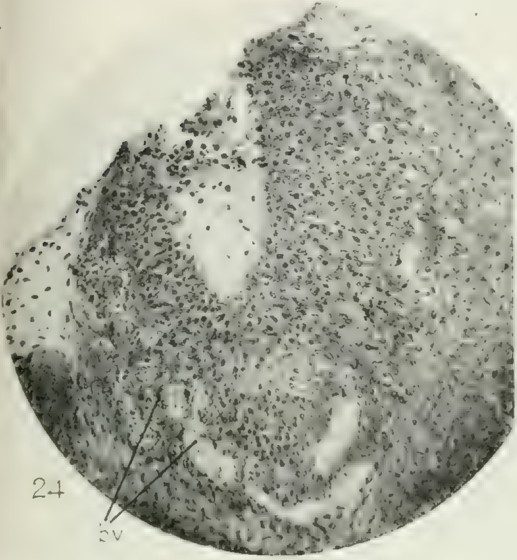
EXPLANATION OF FIGURES

24 A section of the ovary from 1086 ♀ showing a follicle shortly after rupture. The congestion in the theca folliculi is evident. *bv*, blood vessels. This animal was killed at the end of the second stage or early beginning of the leucocytosis, the third stage.

25 A degenerating atretic follicle from the same ovary as figure 24, the cells of the cumulus oöphorus are degenerating while the follicle is being invaded by leucocytes. The ovum shows the first polar body in process of division while the nucleus of the egg is represented by a small chromatic mass near the center.

26 An early corpus luteum from animal 1104 ♀ killed during the third stage. Near the corpus luteum is seen a degenerating atretic follicle invaded by leucocytes. Compare smears figures 5, 6, 7 and 8, and sections of uterus figures 15 and 16, and section of vagina figure 17, all from the same animal.

27 A somewhat older corpus luteum from 1099 ♀ killed during the hemorrhage stage. The vascularization of the corpus is apparent at the periphery and is growing toward the center. *bv*, blood vessels. Compare the smear in figure 9, and section of the uterus figure 18.



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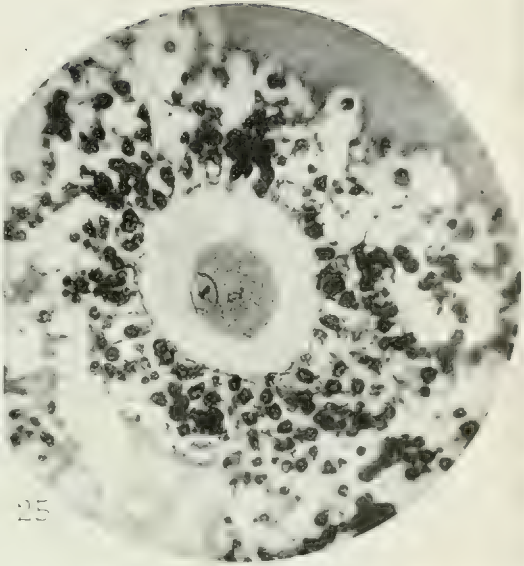

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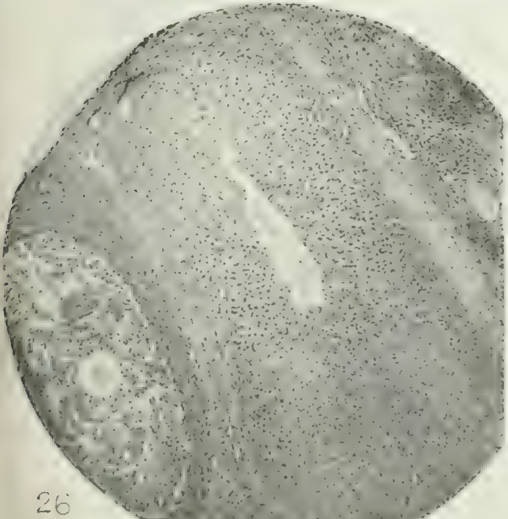

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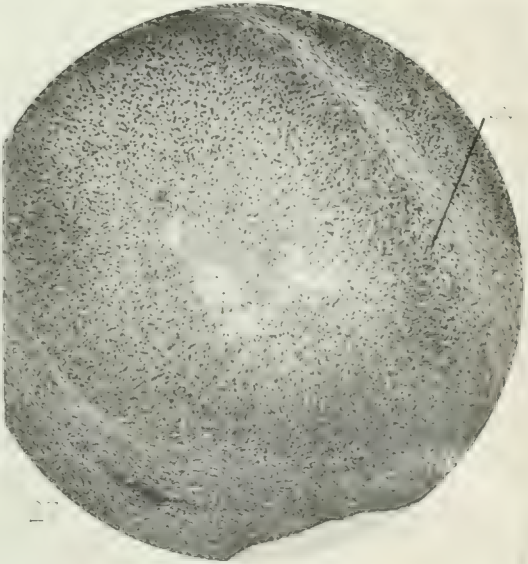

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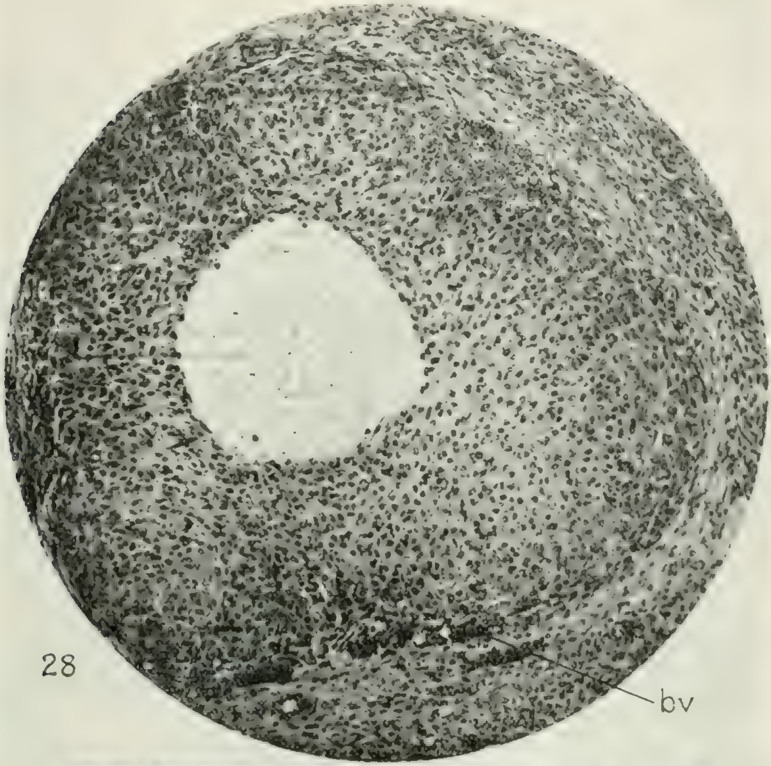
PLATE 9

EXPLANATION OF FIGURES

28 A higher magnification of another corpus luteum from the same ovary as figure 27. The ingrowth of the peripheral vessels is more apparent, *bv*, blood vessels.

29 A fully developed corpus luteum from animal 1074 ♀, killed four and one-half days after oestrus. The typical glandular structure is clearly shown, cords of cells surrounded by capillaries. *cap*, capillaries. A section of the wall of the resting uterus from the same animal is given in figure 10.



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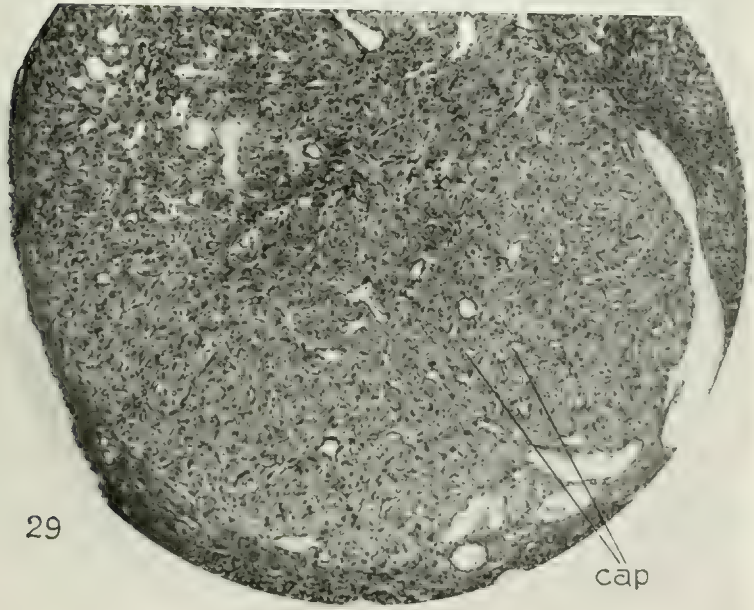

29

Reprinted from The Anatomical Record, Vol. 11, No. 6
January, 1917

*59. *The morphological changes of the idiosome during the spermatogenesis of the guinea-pig.* C. R. Stockard and George N. Papanicolaou, Cornell Medical School, New York City.

La Valette St. George was the first to describe the idiosome under the name 'Nebenkern' in 1865–1867. Since then this body has been described under many different names in a great number of papers treating the processes of spermatogenesis and oögenesis in different animal classes. Meves ('99), described the structure during the spermatogenesis of the guinea-pig and called it the 'idiozom' (from idios—own, and zoma—belt-). Regaud ('10) proposed a modification of this term to idiosome (from idios own and soma-body). We accept the modification as better fitting our conception of this structure.

Not very much specific study has been devoted to the idiosome probably on account of the fact that its close position in relation to the centrosomes has made some observers fail to realize that it is in independent body, having its own peculiar history. There is certainly at particular stages a very close relation as to position between the idiosome and the centrosomes, but the relation is temporary. In only one stage in the spermatogenesis of the guinea-pig, are the centrosomes really in very close connection with the idiosome, and this is in the primary spermatocytes. During this stage the centrosomes are enclosed in the center of the idiosome. But as soon as the spireme begins to form they come out of the idiosome to play their usual rôle during the nuclear division, while the idiosome continues its independent development with a surprising succession of highly specialized morphological changes. In the later stages, the secondary spermatocytes and spermatids, no connection is to be observed between the centrosomes and the idiosome.

The idiosome in the male germ cells of the guinea-pig is present even in the spermatogonia. At this stage the idiosome is of somewhat irregular shape and appearance and its internal structure is not clear and definite. First in the primary spermatocytes, the idiosome takes a regular spherical form and shows a clear differentiation into two zones, a peripheral, the idioectosome, and a central, the idioendosome. The latter zone is completely enclosed by the former. When the prophase begins in the division of the primary spermatocytes the idioendosome breaks up into a number of granules the idiogranulomes. During the progress of division, the idioectosome also breaks into smaller pieces, which, together with the idiogranulomes are dispersed throughout the protoplasm. In this way a uniform distribution of the idiosomatic material is secured during the division process.

At the end of the division the idiogranulomes and the pieces of the idioectosome begin to flow together near the daughter-nuclei and thus form two daughter-idiosomes. In the secondary spermatocytes the idioectosome takes a regular spherical shape, while the idiogranulomes form a group of closely arranged granules in its center, as if they were preparing to fuse. A fusion does not take place, however, probably on account of the short existence of the secondary spermatocytes.

During the division process of the secondary spermatocytes the idioectosome again breaks up into small pieces to be distributed in the cytoplasm together with the liberated idiogranulomes. In this way repeating the phenomenon which occurred in the primary spermatocytes. In the telophase a new flowing together of the idiogranulomes and of the pieces of the idioectosome takes place near the new daughter-nuclei, forming the idiosomes of the spermatids.

The idiosome of the spermatids thus has the same formation as that of the secondary spermatocytes, consisting of a large idioectosome containing a great number of small idiogranulomes. The idiogranulomes are each enclosed in a small vacuole, the idiogranulotheca. Soon after the spermatids are formed the idiogranulomes and idiogranlothecae fuse together into larger and larger masses and vacuoles until finally they form a single big body, the idiosphaerosome surrounded by a large vacuole the idiosphaerotheca. The idiosphaerosome, as soon as formed, begins to secrete on its surface furthest from the nucleus a new substance, showing a different color reaction and a vacuolar consistency. This substance, the idiocalyptrosome, covers in a cap-like fashion the remains of the idiosphaerosome now called the idiocryptosome. At this time the idioectosome forms a cap above the idiosphaerotheca and later on when the secretion of the idiocalyptrosome is about complete the idioectosome becomes detached as a separate body. It then moves along the nuclear membrane and finally goes over into the remains of the protoplasm on the posterior pole and is eliminated.

In later stages of development the idiocryptosome comes to lie in the form of a small cap on the anterior pole of the nucleus. The calyptrosome grows into a very large body, always showing a vacuolar consistency. In a still later stage, when the spermatid comes into connection with a Sertoli cell, the idiocryptosome and the idiocalyptrosome are elongated in the form of two cones, the one enclosed by the other. During the final metamorphosis of the spermatid, the cryptosome again assumes the form of a cap, covering the anterior part of the head of the sperm, while the calyptrosome loses its original vacuolar consistency and forms an outer larger cap covering over the inferior cryptosomal cap. The calyptrosome cap and part of the head of the spermatozoön are covered by the idiocalyptrotheca, which is the fully developed and transformed idiosphaerotheca.

A point of special interest is the appearance of small granules, comparable to the idiogranulomes, in the nucleus of the germ cells during all stages of development. These we have termed caryogranulomes. Such granules are to be seen in the nucleus of the spermatogonia, the primary and secondary spermatocytes and the spermatids. The caryogranulomes are usually of small size about that of the smallest idiogranulomes, but in some stages they also seem to fuse together forming large granules. Their appearance, their color reactions and their tendency to fuse all show great similarity to the idiogranulomes, yet no genetic relation was directly observed. The caryogranulomes persist up to the time of the metamorphosis of the spermatid into the spermatozoön. Then they dissolve in the head of the sperm in the same way as does the chromatic material of the nucleus.

The new points brought out by this study are the following: First, The recognition of the idioendosome. Second, The description of the formation of the idiogranulomes through the breaking up of the idioendosome. Third, The persistence and the behavior of the idiogranulomes during the divisions of the primary and secondary spermatocytes. Fourth, The existence of the caryogranulomes and their development. Fifth, The exact manner of the formation of the calyptrosome and its vacuolar structure. Sixth, Certain peculiarities in the development of the cryptosome. Seventh, The double nature of the idiosome, consisting of an ectosomatic and an endosomatic substance, each having an independent development. Eighth, the rôle of the granulation of the idioendesome in serving to distribute the idioendosomatic substance during each division.

Reprinted from The Anatomical Record, Vol. 11, No. 6
January, 1917

*58. *The existence of a typical œstrous cycle in guinea-pigs and its histology.* CHARLES R. STOCKARD and GEORGE N. PAPANICOLAOU, Cornell University Medical College, New York City.

Normal guinea-pigs of our control stock possess a regular periodic procœstrum, occurring every fifteen or sixteen days. This fact was ascertained by examining the vaginae by means of a speculum every day during different seasons of the year. The flow, which marks the procœstrum activity, is not very abundant and consists largely of desquamated epithelial cells and some mucous secretion.

In the first stage there is a flow of a mucous fluid filled with superficial squamous cells of the vagina. A few hours later a thick cheese-like substance occurs in the vagina. This consists almost entirely of the deeper epithelial cells which preserve their eipthelial structure and often remain together in groups or actual pieces of epithelial tissue. This thick vaginal substance after a few hours becomes more fluid in consistency and pus-like in appearance. A microscopical examination at this time shows a very large amount of polymorphonuclear leukocytes among the epithelial cells and the beginning of an active phagocytosis. The result of this phagocytosis is that in a few hours the vagina is almost completely cleaned and no longer contains the menstrual substance except for a little fluid containing leucocytes and a few broken down epithelial cells. The occurrence of either red blood corpuscles or hemoglobin in the menstrual flow takes place only in the later stages.

The active phagocytosis may probably account for the fact that none or very little of the pus-like fluid ever flows out from the vagina. The leucocytes, migrating from the subepithelial capillaries of the uterus and the vagina, as microscopical sections show, attack the desquamated epithelial cells within the lumen of the uterus and vagina and there begin to destroy them. The entire process of menstruation is not long, its duration being less than twenty-four hours. But during this time the entire genital tract is inflamed by a very active circulation of blood.

The anœstrous period of the uterus and vagina is characterized by the absence of the secretions and the constant presence of leucocytes. Especially the first week after procœstrum, the vagina is very clean and dry. During the second week and particularly a few days before the next procœstrum there is a little mucous fluid in the vagina, and this contains some leucocytes and a few squamous cells. The massive desquamation and the abundant thick secretion, however, occurs very regularly every fifteenth or sixteenth day.

The menstruation or procœstrum seems to be closely followed by an ovulation. At this stage of our preliminary study of the correlation between menstruation and ovulation observations indicate that the ovulation occurs about eighteen hours after the height of menstruation (the presence of the abundant thick secretion).

[*Reprinted from* SCIENCE, *N. S., Vol. XLVI., No. 1176, Pages 42–44, July 13, 1917*]

A RHYTHMICAL "HEAT PERIOD" IN THE GUINEA-PIG

DURING the past six years we have been using guinea-pigs in an extensive breeding experiment and it has become more and more evident as our work goes on that the existing notions of the ovulation periods in these animals are of no practical value, or are practically incorrect. In a number of the experiments it became important to know accurately when the females "came into heat" and when ovulation took place. We had concluded, from numerous observations as well as theoretically, that the female guinea-pig very probably had a definitely regular and periodic sexual cycle if it could be worked out exactly. On account of the need of this exact information, we have studied the œstrous cycle in these animals during the past eighteen months.

Most other attempts at a solution of this problem have centered in a study of the ovary, which necessitated either its removal by operation or the killing of the animal. In either case the procedure brought to a conclusion the observation or experiments on the ovulation cycles in that specimen. Recognizing, on the other hand, that no thorough investigation of the uterus and vagina in the living female had been made, it occurred to us that possibly œstrous changes might take place even though they are so feebly expressed as not to be noticeable on casual observation. The absence of an apparent œstrous or proœstrous flow from the vagina of the guinea-pig has, no doubt, been the chief reason for the general lack of knowledge of the œstrous cycle. It was, therefore, determined to make a minute examination of the contents of the vaginæ of a number of females every day for a long period of time to ascertain whether a feeble flow might exist, although insufficient in quantity to be noticed at the vaginal orifice or vulva.

The observations were made by using a small nasal speculum which was introduced into the vagina and the arms opened apart by means of the thumb screw. This instrument permits an examination of the entire surface of the vaginal canal. In this way the vaginæ of a number of virgin females have been examined daily and smears made from the substances that happened to be present in the lumen.

By the use of such a simple method, it was readily determined after examining the first lot of animals for a few months that a definite sexual period occurs lasting for about twenty-four hours and returning with a striking regularity every fifteen or sixteen days. During this twenty-four hour period the vagina contains an abundant fluid which is for about the first half of the time of a mucous consistency. The vaginal fluid then changes into a thick and cheese-like substance which finally becomes slowly liquefied and serous. This thin fluid exists for a few hours and then disappears. Occasionally toward the end of the process a slight trace of blood may be present, giving the fluid a bloody red appearance, otherwise it is milk-white or cream color.

According to the changes in appearance and consistency of the vaginal fluid, one may distinguish four different stages. The first stage having a mucous secretion, a second stage the cheese-like secretion, a third stage with the fluid becoming serous and a fourth stage, not always recognized, during which a bloody discharge is present. The duration of these several stages is subject in the different animals to individual variations. The first stage, however, is generally longest and lasts from six to twelve hours or even more, and during this time there is a gradually increasing quantity of the mucous secretion which at its height is very abundant and fills the entire lumen of the vagina. The second stage is shorter, lasting from two to four hours, and passes gradually over into the third stage which lasts from four to six hours. The fourth stage is the shortest, only about one to two hours long, and for this reason it is often missed in examining the animals during the periods. It is also possible, as mentioned above, that the fourth stage may not typically exist in all individuals and the quantity of blood present is very different in the different specimens. The succession in which these stages follow one another is remarkably definite. We have never observed any change in the typical sequence of the stages and the time consumed by the entire process is closely the same in all cases.

A macroscopical examination of the uterus and vagina during this period of sexual activity shows the entire genital tract to be congested. The vessels to the ovary, uterus and vagina are large and conspicuous, the uterine horns and the vagina are slightly swollen and inflamed. However, as soon as this short period of activity is over, the congestion disappears and the uterus and vagina take again their normal pale aspect. At the same time the vaginal fluid diminishes and the vagina, especially during the first week after the sexual activity, is as clean as possible, showing none of the secretion. The external vaginal orifice, which during the period of activity is more or less open, actually showing in a few cases a little fluid or some blood, closes and becomes less accessible after the period.

During the second week following œstrus a little mucous discharge begins to appear in the vagina and increases progressively, indicating that the new period of activity is nearer and nearer approaching. The orifice of the vagina is sometimes open during this stage and thus explains why this sign, which was observed before, does not make it possible to detect the actual time of the regular œstrous activity.

The complete results of the present study which will be published in full elsewhere may be stated in brief as follows.

Guinea-pigs kept in a state of domestication and under uniform environmental conditions possess a regular diœstrous cycle repeating itself in non-pregnant females about every sixteen days throughout the entire year with probably small and insignificant variations during the different seasons.

During each cycle typically corresponding changes are occurring in the vagina, the uterus, and the ovary; a given stage in one of these organs closely accompanying parallel stages in the other two.

Each period of sexual activity lasts about twenty-four hours and is characterized by the presence of a definite vaginal fluid, which is not sufficiently abundant to be readily detected on the vulva, but is easily observed by an examination of the interior of the vagina.

The composition of the vaginal fluid changes with the several stages of change occurring in the uterus and vagina.

(*a*) To begin with, during what we term the *first stage,* the fluid consists of an abundant mucous secretion containing great numbers of desquamated vaginal epithelial cells. At this time sections of the vagina show an active shedding or desquamation of its epithelial lining cells. The cells of the uterine epithelium are loaded with mucus, and an active migration of polynuclear leucocytes is taking place from the vessels of the vagina and uterus out into the stroma and towards the epithelial layer.

(*b*) During the *second stage* the contents of the vagina become thick and cheese-like on account of the great accumulation of desquamated epithelial cells. The walls of the uterus and vagina become congested and the migration of leucocytes becomes still more active.

(*c*) The leucocytes reach the epithelium and vigorously invade its cells and intercellular spaces during the *third stage.* These wandering cells become enclosed within and apparently dissolve the breaking-down dead cells of the epithelium. The vaginal fluid becomes thinner under the dissolving or digesting action of the leucocytes. The congestion in the uterus and vagina becomes still more pronounced, giving rise to small blood masses or hæmatomata beneath the epithelium. The epithelium of the uterus is highly disorganized, vacuolized and richly invaded by the leucocytes, so that portions of it fall away en masse, actually carrying with it in some cases cells of the stroma.

(*d*) The *fourth stage* is merely a continuation or result of the activities of the third. The falling away of the epithelial pieces and stroma cells permits the escape of the small hæmatomata or blood knots, thus causing a slight bleeding into the lumen of the uterus and vagina. These traces of blood often give a reddish aspect to the vaginal fluid. At this same stage a regeneration process begins from the necks of the uterine glands and also apparently from the epithelial infoldings in the vagina, so that the lost epithelium becomes rapidly replaced almost before it has ceased falling away.

The regeneration process in the guinea-pig is very short, lasting only a few hours, from six to twelve in all.

Ovulation seems to occur spontaneously during every heat period without exception. The rupture of the follicles with the consequent ovulation takes place about the end of the second stage or the beginning of the third; that is, during the presence of the thick cheese-like vaginal fluid.

During the diœstrum or intermenstrual period there is very little fluid to be found in the vagina. This scant fluid consists of mucus in which are some atypical squamous cells from the vaginal wall and many leucocytes. A number of the leucocytes are old but there are probably new ones arriving almost continuously from the wall of the vagina. The only time at which the vagina seems to be practically free of leucocytes is immediately before and during the first and second stages of the œstrous period described above.

A marked correlation exists between the œstrous changes in the uterus and the developmental cycle of the corpora lutea. When the corpora lutea are highly developed and apparently active the mucosæ of the uterus and vagina show a normally vigorous and healthy condition. While on the other hand, when the corpora lutea begin to degenerate during the second week after the "heat period" the mucosæ of the uterus and vagina also begin to shown signs of degeneration and the process of desquamation slowly commences. At about two weeks after the last "heat period," when the wholesale destruction of the mucosa begins, the corpora lutea are almost completely degenerated. The breaking of the Graafian follicles occurs during the œstrus as a result of a congestion which began in the theca folliculi at about the same time as the congestion of the stroma of the uterus and vagina. And finally when the regenerative growth of the uterine mucosa sets in, the ovaries then possess new corpora lutea in an active state of differentiation which were derived from the recently ruptured follicles.

It, therefore, might be imagined that the secretion from the corpora lutea exerts a protective influence over the uterus and vagina while the absence of this secretion permits the breaking down and degeneration of the uterine epithelium typical of the "heat period."

C. R. Stockard,
G. N. Papanicolaou

[Reprint from INTERSTATE MEDICAL JOURNAL, Vol. XXIII., No. 6, 1916.]

THE HEREDITARY TRANSMISSION OF DEGENERACY AND DEFORMITIES BY THE DESCENDANTS OF ALCOHOLIZED MAMMALS.

By CHARLES R. STOCKARD, Ph. D., of New York,
Professor of Anatomy, Cornell University Medical College, New York.

INTRODUCTION.

A little more than two years ago the author* recorded experiments which had then been running for three years and seemed to show a definite injury of the germ cells by treating mammals with the fumes of alcohol. This injury of the male germ cells is of such a nature that an alcoholized male guinea-pig almost invariably begets defective offspring even when mated with a vigorous normal female. At that time it was also shown that F_1 animals, the offspring of treated parents, though themselves not treated, had the power to transmit the defective condition to their young, and such F_2 young were equally if not more defective than the immediate offspring of the treated animals.

In 1914 in a short abstract I showed further** that the offspring from F_2 individuals were apparently more defective than their parents and were often grossly deformed. One case was recorded of the occurrence of a litter of two F_3 animals, both of which were extremely weak and neurotic, showing a condition suggesting paralysis agitans, and further than this the two animals were typical anophthalmic monsters. The eyes were completely absent, no optic nerve or optic chiasma or visible optic tracts along the tuber cinereum could be found on a careful gross examination of the brain. The two animals were produced by parents (F_2) that had never been treated with alcohol, the four grandparents (F_1) had also not been treated, while the *three great grandfathers* had been alcoholized and the three great grandmothers were normal untreated individuals.

Defective eyes and absence of one eye or both eyes have been frequently met with in the experiment, as well as the peculiar nervous condition, and these symptoms are to be considered indicative of the injury or change induced in the male germ cells by the experimental treatment, which in the above case was transmitted through

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*The Effect on the Offspring of Intoxicating the Male Parent and the Transmission of the Defects to Subsequent Generations. (*Amer. Naturalist*, 1913, Vol. XLVII, p. 641.)

**A Study of Further Generations of Mammals from Ancestors Treated with Alcohol. (*Proc. Soc. Exp. Biol. and Med.*, 1914, Vol. XI, p. 136.)

three generations. No question could remain as to the action on the germ cells, as only male ancestors had been treated; every female of the line was an untreated animal.

This abstract called attention to the fact that there was a tendency for the results to differ in subsequent generations from treated males as compared with the descendants of treated females; but not enough data were then present to offer any explanation of these differences and a consideration of them will be undertaken in the present paper.

At that stage of the experiment it was also difficult to offer an exact analysis of the mode of transmission of the defects and the type of injury induced by the alcohol treatment, since the total numbers were not large and the F_2 animals had only a few matings, while further generations had not become available for breeding.

The same experiments have now been continued for more than five and one-half years and a number of animals have been used, over 1,300, which cover the behavior of five generations and supply data of sufficient extent to allow a more thorough analytical consideration of the heredity problem concerned.

Experiments of this nature on mammals are fraught with many difficulties, slowness of breeding, small size of litters, difficulty of handling, etc. Yet such material offers one very great advantage in that the quality of the offspring and generations studied is of such a complex that one is enabled to detect indications of rather slight injuries or changes in the material carriers of heredity which would not become evident on lower forms with less diversity in their methods of behavior and structural appearance. In other words, we take it that such conditions as are spoken of as racial degeneracy in man and mammals are often very difficult or even at times impossible to detect in lower forms.

These conditions are for many reasons thought to be inherited. If so, their inheritance must be due to a pathological condition of the material carriers of heredity, the chromosomes, or what not, since they are not normal states and, like diseases, are constantly arising in normal families on account of one or another form of intoxication. Is it possible then to produce such a racial degeneracy artificially by treating only one generation of the animals and by so doing observe a pathological behavior of the carriers of heredity? Arguing from analogy there must be pathological heredity due to diseased or altered chromosomes in the germ cells just as truly as there is a known pathological behavior of every other organ and tissue of the animal body.

It becomes then a problem to study the possible methods of modifying the chromosomes or carriers of the inherited qualities of organisms in order further to analyze their normal physiological behavior; in the same way that experimental embryology has been able to supply so many valuable clues to the normal processes of development.

In the following pages we believe the facts indicate that individual guinea-pigs are now living in this experiment that have had the carriers of hereditary qualities, the chromatin, of their germ cells injured for a longer time than five years. And during this time they have given rise to offspring of more or less degenerate or deformed type, and in some cases these offspring have passed this modified chromatin on through three generations, all of which contain pathological chromatin and show some somatic defects and deformities as an index of their tainted chromatic ancestry. Modified chromatin has been living in the experiment for more than five years in six different generations of animals as a result of the alcohol treatment on the one original, P_1, parent generation.

We have tried to regulate every controllable source of error, and there can be no doubt that the conditions are brought about in the way described. Could the degeneracy which is so pronounced have previously existed in the stock? This question has been controlled in the first place by the use of two entirely different stocks from different sources and obtained one and one-half years apart, the first in the fall of 1910 and the other in the early winter of 1912. The responses of the two stocks to the experimental treatment have been identical. As a second method of control every animal has been tested by one or more normal matings before being introduced into the experiment, and only those giving normally strong offspring have been used. A further crucial control is the constant mating of normal untreated animals from both stocks under identical cage conditions with the experimental individuals. These animals continue to breed normally until very old, when they gradually become sterile. But none has ever given rise to a defective or deformed individual, and the rate of mortality of the young indicates the average healthy condition found in normal guinea-pig breeding. There is a striking contrast between the records of these normal young and the mortality record, the frequency of easily recognized nervous symptoms of degeneracy, and the prevalence of gross deformities in the experimental alcohol races.

The external as well as internal factors are to be considered not only in individual or embryonic development, but also in heredity. And the present experiments now demonstrate for mammals that either the spermatozoon or the ovum may be experimentally injured or modified by alcohol in such a manner as not only to give rise to (abnormal) subnormal development in the resulting embryo, but the effects of the injury may be transmitted from generation to generation, until an affected line actually fades out through degeneracy and sterility as a result of the transmitted condition.

MATERIAL AND METHODS.

The animals used in the experiments have been ordinary vigorous guinea-pigs of large size, particular care being taken to select animals less than one year old to begin with and good breeders.

At the beginning of the experiments alcohol was given along with the food, but the animals ate less and the food usually disagreed with them. It was then administered in diluted form by a stomach tube; this method was even more unsuccessful, disturbing digestion and seeming to upset the animals considerably. It is certain that alcohol given to animals through the stomach deranges their appetite and digestion to such an extent that the experimenter is unable to determine whether the resulting effects are due to the alcohol, as such, or to the generally deranged metabolism of the animal. When given in drinking water they take little or none of the water and the treatment is insufficient. For these reasons an inhalation method of treatment was resorted to early in the study, and, as far as experience goes, it has no serious disadvantages and does not complicate the conditions of the experiment.

This method may be merely described in brief for the convenience of the reader, since it has been fully recorded with illustrations of the fume tanks in previous publications. A fume tank of copper is made of sufficient size to supply breathing space for four or five guinea-pigs at one time. The tank has four outlets, so that a definite amount of fumes may be passed through in a given time and the ventilation controlled. In this way each animal could be given a definite measured dose. The individuals, however, differ so much in their resistance to the treatment that it has been found better to treat all to about the same degree of intoxication. Such a physiological index is more reliable, since every animal may be affected to the same degree each day. For this purpose the animals are placed in the fume tank on a wire screen, and absorbent cotton soaked with alcohol is placed beneath the screen, so that they inhale the alcohol fumes arising from the cotton to saturate the atmosphere of the tank.

Ether was given in a similar manner. The animals are much more readily overcome by these fumes and must be carefully watched while inhaling even the most dilute doses.

To avoid handling the females during pregnancy, special treating cages are devised. An ordinary box-run with a covered nest in which the animal lives is connected by a drop-door with a metal-lined tank, having a similar screen arrangement, etc., to that of the general treatment tank. The pregnant animal may be driven daily into the tank and thus treated with alcohol fumes throughout her pregnancy without being handled in any way that might disturb the developing fetus.

Particular care is necessary in mating the animals in such an experiment, as the females are often slow to conceive and some of the F_2 and F_3 individuals of both sexes are not very prolific and in many cases are almost or quite sterile. Each female is kept in a separate run and the male is placed in with her just before the time of the expected heat period, ovulation, and he remains in her cage for from two to three weeks so as to be present at the second ovulation, provided the female was not made pregnant by the first mating. The ovarian cycle of the guinea-pig as worked out by L. Loeb seems to correspond closely to what is found in mating experiences.

After mating, the male is removed from the cage and the female remains alone until the young are born. These are left with the mother for about fifteen days, then separated, and the female mated again. In this way the normal females may sometimes give as many as four litters per year, but the experimental animals breed much slower and it is difficult to get even three litters per year.

Direct Effects of the Alcohol Treatment on the Animals.

Several of the guinea-pigs have now been treated with the fumes of alcohol almost to the point of intoxication for six days per week for a period of five years. This is a considerable space in the life of a guinea-pig, which probably would not often extend beyond six or seven years.

The animals are affected by the alcohol fumes in various ways; some of them are stupefied and become drowsy, while others become stimulated and excited and sometimes even vicious, constantly fighting and biting at the other animals in the fume tank. The fumes inhaled into the lungs pass directly into the circulation, so that the animals show signs of intoxication very soon after being put into the tank, yet the intake of alcohol is so gradual that they may remain for one hour or more without becoming totally anesthetized.

The mucosa of the respiratory tract is considerably irritated during the early stages of the treatment, but develops a resistance so that later little effect can be noticed. The cornea of the eye is greatly irritated, often becoming milky white and opaque during the first few months. In some cases this later clears and the animal is again able to see, though some of the animals treated for several years have remained entirely blind. The general condition of the animals under the fume treatment is very good. They all continue to grow if the treatment is begun before reaching their full size, and become fat and vigorous, taking plenty of food and behaving in a typically normal manner.

Some of the treated animals have died and others have been killed at different times during the progress of the experiment and their organs and tissues examined carefully and then studied microscopically. All have seemed practically normal. Tissues from several animals treated as long as three years have been examined and the heart, stomach, lungs, kidney, and other organs present no noticeable conditions that might not be found in normal individuals. Alcoholized animals are usually fat, but there is no fatty accumulation in the parenchyma of any of the organs.

Several of the animals, both males and females, have been partially castrated during the experiments and the ovaries and testes have been found to be in a healthy condition, though certain possible changes may be present which are now being closely studied cytologically and experimentally.

The treated animals are, therefore, little changed or injured so far as their behavior and structure go. Nevertheless, the effects of the treatment are most emphatically shown by the type of offspring to which the alcoholized individuals give rise, whether they be mated together or with normal individuals. The further significance of the nature of the effects is indicated by the quality of the subsequent generations descended from such an ancestry.

INFLUENCE OF THE TREATMENT ON THE DESCENDANTS OF ALCOHOLIZD ANIMALS.

The records of the matings of the alcoholized animals in various pairs, the control or normal matings, and the matings of the F_1 and F_2 generations, the children and grandchildren of the alcoholized individuals are summarized in the general Table. This table gives a record of all the matings of the kinds indicated up to March 24th, 1916. A similar table was published two years ago, when the number of animals considered was much smaller and the actual indications from the results were less certain than now. On comparing this table with the former one, however, it will be seen that the continuation of the experiments has fully substantiated the results as previously recorded. The table now shows the records of 887 matings which produced 1,115 full-term young and 288 early abortions or negative results. These numbers are now of considerable magnitude in spite of the fact that the experiment is conducted on mammals which produce only small litters and breed slowly as compared with lower animal forms.

In the first horizontal line the record of pairing alcoholized male guinea-pigs with normal females is given. This combination could only produce defective or subnormal young as a result of the injured male germ cells, since the ova are normal and develop in a normal untreated mother. This then is the definite test of the influence of the alcohol treatment on the germ cells.

Ninety-five such matings have in 38 cases given negative results; that is, failures to conceive, or early abortions. Thus 40 per cent. of the matings of such males were non-productive, while less than 22 per cent. of normal matings under the same breeding conditions

Table

Effects of Alcohol on the Descendants of Treated Animals

March 24, 1916

Condition of the Animals	Number of Matings	Negative result or early abortion	Still-Born Litters	Number of Still-born young	Living Litters	Youngdying soon after birth	Total Dead	Surviving Youngs
Alcoholic ♂ x Norm ♀	95	38 40%	10 10%	20	47 50%	1CF 4Pa 39	59	2CE 52
Norm ♂ x Alcoholic ♀	43	11 28%	7 16%	20	25 56%	1P 26	46	26
Alcoholic ♂ x Alcoholic ♀	42	20 48%	4 10%	8	18 42%	12	20	15
Summary	180	69 38%	21 12%	48	90 50%	77	125	93
Control Norm ♂ x Norm. ♀	123	26 21%	2 1.6%	8	95 77%	24	32	154
♀ Treated during Pregnancy	4	0	0	0	4	1	1	7
2n Generation x Norm.	55	13 24%	3 5.4%	6CF 8	39 71%	2par 31	39	3CF 35
2n Gener x Alcoholic	57	16 28%	9 16%	1DE 22	32 56%	2DE 25	47	31
2n Gener x 2n Gener	111	34 30%	8 7.2%	18	69 62%	5CTE 4Pa 46	64	1DF 1onet 2CE 1eyeless 70
3d Gener. x 3d Gener.	62	23 37%	7 11%	1Def 14	32 52%	2 eyeless 6 par 1 D.F 31	45	23
3d Gener x 2n Gener.	47	17 36%	5 11%	9	25 53%	2 par 4 Def 21	30	24
3d Gener x Normal	26	8 31%	5 19%	9	13 50%	6	15	9
3d Gener x Alcoholic	6	1 66%	0	0	5	4	4	6
2n 3d Gener x 2n 3d Gener	36	12 33%	3 8%	10	21 58%	1pa 14	24	19
	887	288					551	564

failed to produce full-term litters. Ten stillborn litters, each consisting of two young, twenty stillborn young, resulted from the 95 matings. While the 123 control matings gave only two stillborn litters, and in both cases these were unusually large litters of four individuals each, and they were probably dead on account of the fact that the mother could not give normal birth to so many offspring. The stillborn litters by the alcoholized fathers were all ordinary-sized litters of two young. Thus, while about 11 per cent. of the matings of alcoholized males resulted in stillborn litters, only 1.6 per cent. stillborn litters occurred from normal matings. Forty-seven living litters were produced, thus 50 per cent. of the matings gave full-term living young, while 77 per cent. of the normal matings give living litters of young.

The 47 litters from alcoholic fathers contained in all 91 young, and 39, or almost 43 per cent., of these died soon after birth, while 95 similar litters from the control lost only 24 young, or 13 per cent., out of 178 individuals. Finally, then, from the 95 matings of alcoholic males with normal mates, only 57 full-term litters resulted, consisting in all of 111 young; 59 of these, or 53 per cent., died at birth or soon after, and only 52 individuals, or 47 per cent., survived. This was only about half as good record as the 83 per cent. surviving young from the matings of normal animals. Almost all of the offspring were very excitable, nervous animals, 4 were paralyzed, and 3 of them showed gross deformities of the eyes, while no such conditions were found among any of the offspring of normal animals bred under identical conditions.

These records leave no doubt that the alcoholized male guinea-pig is injured in such a way as to induce a decidedly bad effect upon the quality and mortality of his offspring when compared with the records from normal animals.

The second horizontal line of the table shows the results obtained when alcoholized female guinea-pigs are paired with normal males. In this case there is a double chance to injure the offspring. First through the influence of the treatment on the oocytes or the unfertilized ovarian egg, a direct effect on the germ cells comparable to the injury of the germ cells in the case of the treated males considered above. While in the second place, the developing embryo in the uterus of an alcoholized female may be directly affected by the strange substances contained in the blood and body fluids of the mother. Thus a defective individual may be produced as a result of development in an unfavorable environment or as a result of being derived from an injured or defective egg cell.

Forty-three matings of alcoholized females with normal males have in 11 cases, 28 per cent., given negative results or early abortions; this compares very favorably with the records of the control animals. Seven stillborn litters consisting of 20 individuals were produced. This is a record of 16 per cent. stillborn litters against only 1.6 per cent. from normal matings. The alcoholized females gave birth to 25 living litters containing 52 young, and 26, or 50 per cent., of these died, only 50 per cent. surviving against 86 per cent. survivals among the young of similar control litters. The records of the matings of alcoholized females compare very unfavorably with the record of the control matings. Yet the behavior of the alcoholized females is very little, if any, worse than the records shown by the alcoholized males in spite of the double chance the female has to injure her young.

The third horizontal line of the table indicates the results obtained when alcoholized males are paired with alcoholized females. Here there is every chance for the treatment to show its effect. The percentage of early abortions or negative results is very high, about 48 per cent. more than double that of the control matings. Ten per cent. of the matings produced stillborn litters, each consisting of two young. Only 18 living litters were born out of 42 matings, about 42 per cent., against 77 per cent. living litters from 123 control matings. The 18 living litters contained only 27 young, and 12 of these, or 44 per cent., died soon after birth, while but 14 per cent. of the control offspring died out of a total of 178 individuals. The data from the double alcoholic matings are, therefore, extremely bad in the light of normal matings from the same animal stocks bred under exactly the same cage and food conditions.

The fourth horizontal line summarizes the records of all the matings of directly alcoholized animals. In all, 180 such matings have been made, 69 of these, or about 38 per cent., gave negative results or early abortions. Twenty-one stillborn litters occurred, consisting of 48 individuals against only two questionable stillborn litters from 123 control matings. Ninety, or only 50 per cent., living litters were born, consisting of 170 individuals, 93, or 54 per cent., of which survived and 77, or 46 per cent., died soon after birth; in all 125 full-term young died, while only 93, or 42 per cent., of the total 218 full-term young resulting from the 180 alcoholic matings survived. On the other hand, out of a total of 186 full-term young from only 123 control matings, 154, or 82 per cent., survived. The control matings were far more prolific than those of the alcoholized animals and the condition of the young as indicated by the mortality record was far superior to that of the alcholic offspring.

The fifth line records the outcome of 123 control matings which have been scattered through the entire progress of the experiment under exactly the same conditions and from the same animal stocks as the experimental matings. Eighty-six per cent. of the young in the 95 living litters resulting from the matings of normal animals have survived and all are strong, healthy individuals; in not one instance do they show an indication of nervous degeneracy or any

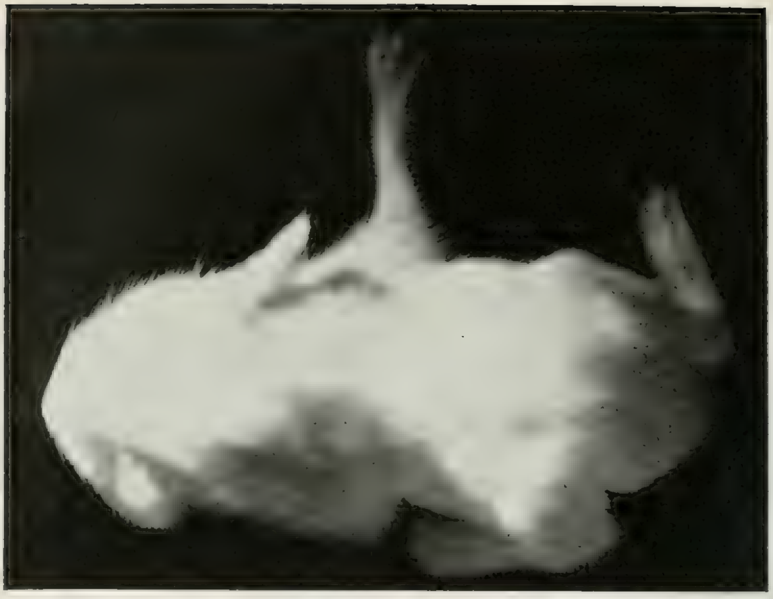

FIG. 1.—521 albino F_2 ♀ (two alcoholic grandmothers, both grandfathers normal). Lived only one day after birth; the meninges of the brain were filled with blood. Gross tremor and complete paralysis of right side. Cataracts, both crystalline lenses being entirely opaque. The photograph shows the outstretched paralyzed right extremities while the left legs are held in a normal position in their effort to support the body. (Birth weight, 54 grm.)

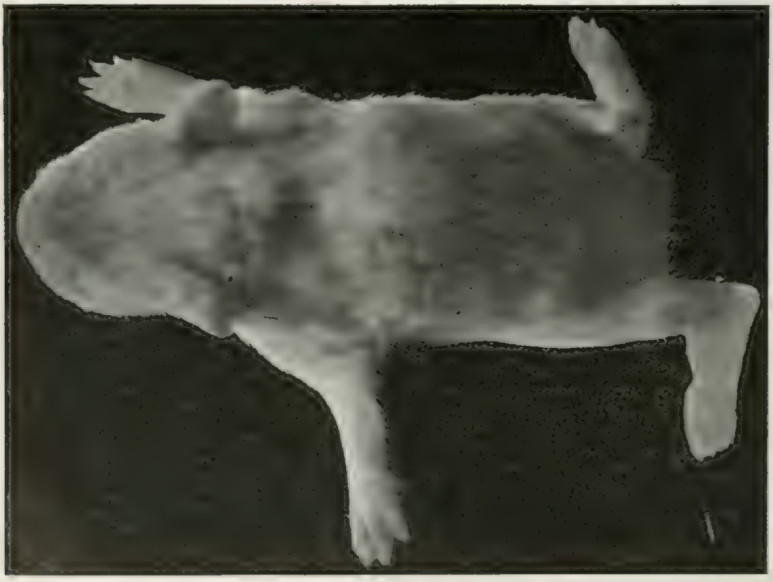

FIG. 2.—506 mouse and yellow $F_{2,3}$ ♂ (two paternal great-grandmothers and the maternal grandmother alcoholic, slightly inbred). Gross tremor and complete paralysis of left side, so unable to walk. Cornea of right eye opaque. Photograph shows the powerless condition of the outstretched left legs with the right legs attempting to support the body. (Birth weight, 57 grm.) The two figures are at different magnifications.

type of recognizable structural deformity, while such degeneracy as well as deformities are extremely prevalent among the offspring and descendants of the alcoholized animals. One other point to be mentioned in considering the records of the control matings is the fact that from 123 matings only two stillborn litters were produced and, as mentioned above, both of these litters were of so large a size that the mothers seemed unable successfully to deliver them and one of the mothers failed to recover from the process and died a few days later. These 2 cases make the control records appear worse than they actually should, but in spite of this the control matings have given data equally as good as those generally obtained by careful breeding experiments with vigorous normal stocks. The stock in these experiments is unquestionably good, as the control matings very readily show.

Four normal females were mated and then treated with alcohol throughout their periods of pregnancy and, as the sixth horizontal line of the table indicates, such a treatment was not at all injurious in these particular cases. It actually happened that some of these young were unusually vigorous. The numbers are very small, but this is a direct test, and if such a treatment were really decidedly effective in its action on the embryo or fetus *in utero* these eight young animals should have at least shown some response. It is very possible that after the treatment has been continued for a long time, a year or more, that the mother then presents a uterine environment unfavorable for normal development, since the offspring of such individuals are almost always subnormal. In these cases, however, the inferior quality of the offspring may be due to the action of the alcoholic treatment on the ovarian germ cells rather than the direct environmental effect on the developing embryo or fetus, since there is no way at such a stage to separate the two possible effects.

The next three horizontal lines, seventh, eighth and ninth, give the data resulting from the matings in various combinations of the F_1 animals, that is, offspring from alcoholic parentage, but which are not themselves treated with alcohol. The records of these non-treated F_1 individuals are most instructive for an understanding of the actual influences of the alcoholic treatments.

When such F_1 animals are paired with normal individuals the seventh line shows that 24 per cent. of the matings failed, which is not a bad record. The proportion of stillborn litters, however, from the F_1 by normal combination was three times as great as from normal matings, and 75 per cent. of the stillborn young produced showed gross defects of the eyes, having opaque lenses or typical cataract conditions, while not one of 186 young from normal matings has shown this or any other noticeably abnormal structure. Thirty-nine living litters were produced containing in all 66 in-

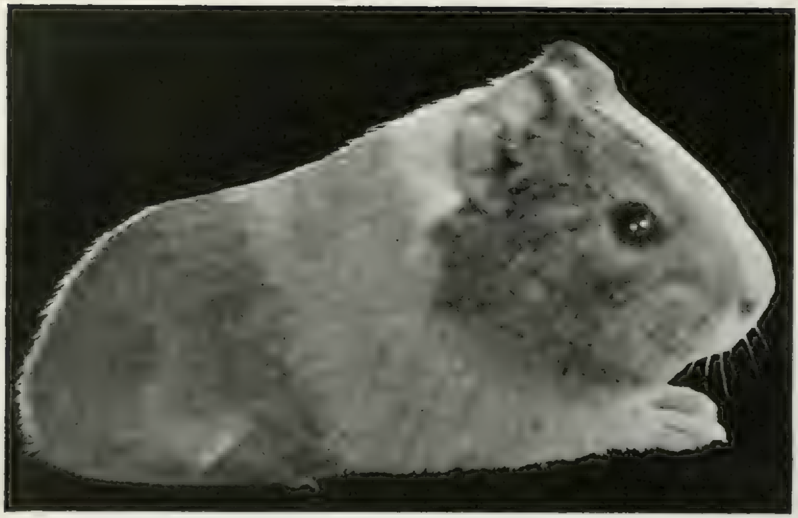

FIG. 3.—701 agouti, yellow and white, normal F_3 ♂. A normal animal from the fourth generation of the control; slightly inbred, natural size. (Birth weight, 63 grm.)

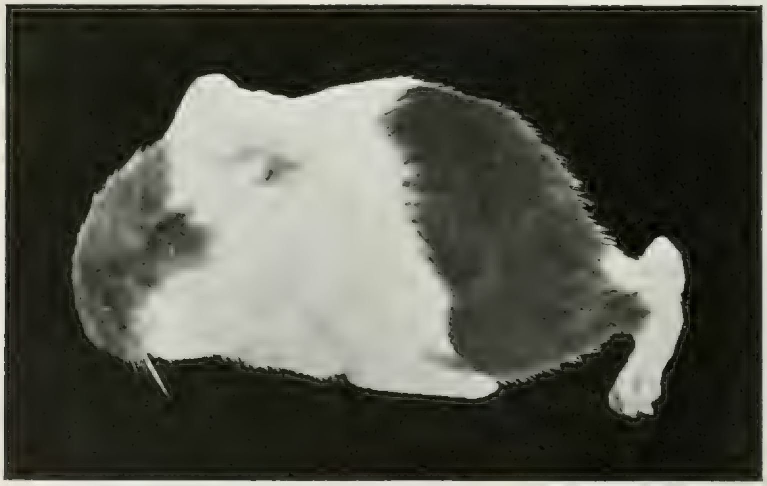

FIG. 4.—599 black, white and red. F_3 ♂. A degenerate animal from the fourth generation alcoholic lines; no inbreeding; the paternal grandmother had both parents alcoholic and the maternal grandfather had both parents alcoholic. So there were two alcoholic great-grandmothers and two alcoholic great-grandfathers; the other four great-grandparents were normal and one grandmother and one grandfather had no alcoholism in their ancestry. The parents were ♂ (NN) (AA) ♀ (AA) (NN). The animal lived seven days and died in convulsions. The photograph shows the front limbs bent under the body and the animal is unable to raise the head. It weighed only 35 grm. at the time of death, having lost 7 grm. While the above normal animal weighed 63 grm., actually a little small, at birth and all normal animals increase in weight rapidly from that time.

dividuals, 31, or 47 per cent., of which died soon after birth, while 35 survived. Two of those dying soon after birth were paralyzed and unable to walk, while three of the 35 survivors have defective opaque eyes, and many show different nervous symptoms. Thus of 74 full-term young produced by F_1 animals with normal mates, only 35, or 47 per cent., survived for more than a short time after birth, and 8 per cent. of these have gross defects and more than half of them are nervous, excitable individuals, which when mated with normal animals or in any other combination always give very poor quality offspring, if any at all.

The eighth line shows the records of 57 matings between F_1 animals and alcoholics. This combination again gives data comparing most unfavorably with the control and in some ways even worse than the records of matings between two alcoholic animals. Sixteen per cent. of such matings produced stillborn litters! Almost half of the young in the living litters died and here again some were deformed. Deformities are strikingly more abundant among the offspring from F_1 and F_2 parents than from the directly alcoholized animals.

The record of 111 *inter se* matings of F_1 animals is shown in the ninth line. Thirty per cent. of such matings gave negative results or early abortions, over 7 per cent. stillborn litters and 62 per cent. living litters. Little less than half of the living young died soon after birth, in all 46, nine of which, or about one in five, 20 per cent., were paralyzed or deformed. Seventy of the offspring survived, five with deformed eyes, one with one eyeball completely absent, monster monophthalmicum asymmetricum, and almost all of the 70 are very nervous, excitable animals which when bred give rise to deformed or highly degenerate offspring.

The offspring from the F_1 animals mated in any combination are generally far below the normal in power to survive and in quality of structure. When compared with the offspring from directly alcoholized parents, the offspring from the F_1 combinations show an equally bad mortality record and a very much higher proportion of paralyzed and deformed individuals. The 111 matings *inter se* of F_1 animals demonstrate conclusively that such individuals carry defective or abnormal germ cells which give rise to defective developmental products. These degenerate F_2 offspring owe their subnormal condition to the effects of the action of the alcohol treatment upon the germ cells of their grandparents which have been transmitted to them through their parents. In other words, the carriers of hereditary qualities have been modified in the first parental generation, and the effects of this modification are expressed in their offspring F_1, and also in their grandchildren, the F_2 generation.

The next line of the table, the tenth, indicates further how the effects of the original modification are transmitted to the great grandchildren or through three generations since the injury. Sixty-two *inter se* matings of F_2 animals gave the results here shown. Almost 37 per cent. of the matings gave negative results or early abortions. About 11 per cent. of such matings gave stillborn litters, 7 in 62 matings, which is remarkably high when compared with any of the above combinations.

Thirty-two living litters were produced, containing in all 54 young; 31 of these, almost 60 per cent., died soon after birth, and only 23 survived. Six of the 31 that died were paralyzed and unable to stand, while 8 of them, a strikingly high proportion, were grossly deformed. Six had one or both eyes deformed and two were anophthalmic monsters, being completely without eyeballs, optic nerves, optic chiasma or any gross signs of optic tracts.

Sixty-eight full-term young were produced by the F_2 matings, but only 23 of these, or just 34 per cent., were able to survive, while about two and one-half times this proportion, or 82 per cent. of the full-term young from control matings, survived as vigorous, healthy individuals. The 23 living F_3 animals are all rather weak and degenerate and almost completely sterile according to a considerable number of careful matings with strong, fertile guinea-pigs. The alcoholic race seems at this stage of the experiment about to fade out in the fourth generation, while normal control lines from the same original stocks have passed far beyond this generation, continuing to breed normally and showing no signs of degeneracy, and never in any case giving rise to a grossly deformed animal.

The eleventh line of the table indicates again the very decided effects transmitted by the descendants of animals which had suffered a modification of their germ plasm by the alcoholization of their tissues.

When F_2 animals are mated with normal individuals the results are very little if any improved over the two above combinations. In this experiment, although one mate was a normal animal, the F_2 mate carried germ cells of so inferior a quality that the output of the combination, admitting the numbers are small, leaves no doubt of the transmission, *through three generations,* of defective conditions induced by alcoholizing the great grandparents of the offspring on only one side of the family, or in only one of the parental lines.

The last line of the table gives the records of mixed combinations of F_1 and F_2 individuals, and here the data are closely similar to those obtained from other combinations of these animals; only about 44 per cent. of the full-term young born are capable of surviving, while 82 per cent. of the control young are living.

Briefly then, a careful study of all these young animals extending over a period of more than five years has afforded data which convincingly show that the treatment of either the male or the female guinea-pig with fumes of alcohol affects the quality of the offspring to which these animals give rise even when paired with normal mates. And further, the changed quality of the offspring is subsequently transmitted through succeeding generations with even more severe marks of degeneration and deformity than those exhibited by the offspring of the directly treated animals.

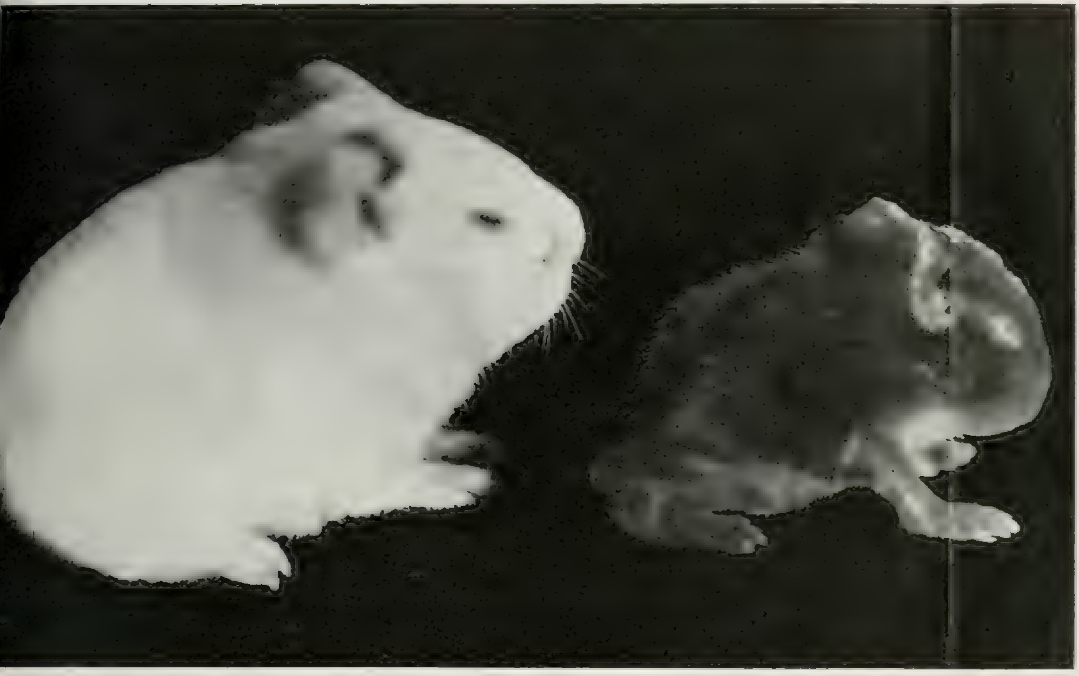

FIG. 5.—955 albino F_3 ♀ and 957 black and red F_3 ♂. Born in same litter, from a normal father and a mother descended from four alcoholic grandparents. The female 955 is a strong animal weighing 90 grm. at birth, taking the characters of the normal father that was also an albino. The male 957 is small, birth weight 38 grm., and degenerate with paralysis agitans and lived only three days, inheriting his degeneracy and color from the black and red mother of alcoholic ancestry.

Other combinations and back crosses are now in progress which are fully in line with the above, but which have not yet afforded sufficient analytical data to record.

The defects caused by the alcohol treatment seem to be largely confined to the central nervous system and organs of special sense. Paralysis agitans is very common among the F_1, F_2 and F_3 animals. Paralyzed limbs are often observed, the animals being unable to stand or walk (Figs. 1, 2 and 4). The eye is also a peculiarly sensitive indicator and presents in the various descendants of alcoholized individuals all degrees of degeneration—opaque cornea, cataract or opaque lenses, small defective eyes, complete absence of one eye, and finally complete absence of both eyeballs—anophthalmic monsters. In the latter case the extrinsic eye muscles, the third, fourth and sixth nerves, the lachrymal glands and other structures of the orbit are present, though the eyeball is completely wanting.

Not only are the above congenital eye defects present, but in several instances members of the alcoholic lines have become blind during the first year or year and a half after birth, whereas in our control this has never occurred.

It is peculiarly interesting to find these particular eye conditions exhibited by the descendants of alcoholized animals, since as I (1910) have previously shown, closely similar eye conditions are obtained in great numbers by directly treating the eggs of fish with solutions of alcohol; and like conditions were also obtained, though not so consistently, by treating hens' eggs (1914) with alcohol fumes either before or during incubation.

SUMMARY OF RESULTS.

In spite of their healthy appearance the injurious influence of the alcohol inhalation is very decidedly shown by the quality of offspring to which the treated guinea-pigs give rise. And the descendants of these offspring are even worse than the F_1 generation when compared with the different generations of control animals produced under identical cage and food conditions.

The males seem to be more injured by the treatment than the females, taking as an index of injury the quality of their offspring and descendants. Stating it differently, the spermatocytes or spermatozoa are more sensitive to the changed chemical condition of the tissues than are the female germ cells.

There is a larger proportion of degenerate, paralytic and grossly deformed individuals descended from the alcoholized males than from the alcoholized females.

The records of 1,115 offspring produced by 887 matings of animals of various types are presented in the accompanying table to show the kinds of litters of young produced and their ability to survive. One hundred and eighty matings of alcoholized animals, in which either the father, mother, or both were alcoholic, gave 69, or almost 40 per cent., negative results or early abortions, while only 21 per cent. of the control matings failed to give full-term litters. Of the 111 full-term litters from alcoholic parents 12 per cent. contained stillborn young, and only 50 per cent. of all the matings resulted in living litters. Forty-five per cent. of the individuals in the litters of living young died very soon after birth. In contrast to this record, 77 per cent. of the 123 control matings gave living litters and 86 per cent. of the young in these litters survived as normal, healthy animals.

The mating records of the descendants of the alcoholized guinea-pigs, although they themselves were not treated with alcohol, compare in some respects even more unfavorably with the control records than do the above data from the directly alcoholized animals.

Of 223 matings of F_1 animals in various combinations 63 have resulted in negative results or early abortions, 20 stillborn litters of 48 young occurred, and 14 per cent. of these stillborn young were deformed. One hundred and forty living litters contained 238 young, but 102 of these died within a few days and almost 13 per cent. of them were deformed, while 136 survived and 8 of these showed eye deformities. Among 186 full-term control young of the same stock not one has been deformed.

The table shows that the records of the matings of F_2 animals, third generation, are still worse, higher mortality and more pronounced deformities, while the few F_3 individuals which have survived are generally weak and in many instances appear to be quite sterile even though paired with vigorous, prolific, normal mates.

The structural defects shown by the descendants of alcoholized animals seem to be confined chiefly to the central nervous system and special sense organs. Many of the young animals show gross tremors, paralysis agitans; the hind legs, fore legs or both legs of one side may be paralyzed (Figs. 1, 2 and 4). Eye defects are very common, such as opaque cornea, opaque lens, various degrees of monopthalmicum asymmetricum, and finally, several cases of complete anopthalmia have occurred, the entire eyeballs, optic nerves and optic chiasma being absent.

The quality of individuals from the same parentage varies inversely with the size of the litters in which they are produced. Animals born one in a litter are rather strong, even though derived from very bad alcoholic lines. This difference between the members of small and large litters is also shown by the normal animals, but the difference in quality between members of large and small litters is ever so much greater in the alcoholic lines. There is also some tendency on the part of the alcoholic animals to produce a greater proportion of small litters and this aids somewhat towards the perpetuation of their lines.

Inbreeding tends to emphasize the alcoholic effects. This is probably due to related animals responding to the treatment in closely similar ways on account of the similarity of their constitutions. Inbreeding, as such, may be harmful. But inbreeding added to the alcohol effects produces a much worse condition in the offspring than either inbreeding or alcoholism alone could do.

The data from alcoholized male lines indicate that the *female offspring from alcoholic males are less viable and more frequently deformed than the male offspring. And heterogeneous matings of* *such male and female offspring further emphasize the same inferiority on the part of the female offspring from treated males.* This is a very significant fact.

The fact that the offspring of one sex differ in quality from those of the opposite sex, and that the female offspring of an alcoholic male are inferior to his male offspring suggests at once a difference between the germ cells concerned in the production of the male and female young. Miss Stevens showed that the spermatocytes of the male guinea-pig contained a heteromorphic pair of chromosomes and half of the spermatozoa would be expected to receive one member, the X chromosome, of the heteromorphic pair and one-half of the spermatozoa the other member, the Y chromosome, of the heteromorphic pair. We now have two possibilities in explanation of the above facts. In the first place, it may be assumed that the alcohol acts similarly on all of the chromatin to injure it. Thus a mass action would cause the spermatozoa carrying the larger member of the heteromorphic pair to deliver more injured chromatin and the other spermatozoa with a less total amount of injured chromatin would deliver less when they fertilize eggs containing equal amounts of normal chromatin. The fertilized egg giving rise to the female, therefore, contains a greater proportional amount of alcoholic chromatin to normal chromatin than does the egg giving rise to the male. And so the female product is actually more injured than the male.

A second possible explanation of these conditions may be that the X and Y chromosomes themselves respond differently to the treatment, the X being the more sensitive of the two. But in either case the two classes of spermatozoa certainly seem to respond differently to the treatment and this shows a physiological difference in behavior to correspond with the well-known morphological differences so often found between the two groups of spermatids of many animal species.

The data from alcoholic female lines indicate that *the male offspring from alcoholic females are inferior in quality to the female offspring. And heterogeneous matings of such male and female offspring further prove the inferiority on the part of the male offspring from treated mothers.* This is also significant. How can it be put in accord with the above chromosomal explanations for the difference in quality between the female and male young of alcoholized fathers?

If we admit that all of the eggs arising from an alcoholized female guinea-pig are homomorphic and contain groups of chromosomes equal in mass, it follows that her male and female offspring receive the same amount of injured chromatin and should be affected by such chromatin to equal degrees. But this is only part of the case; the injured female chromatin is combined with normal chromatin from the normal father when the eggs are fertilized and here the difference arises. The female offspring receive from the normal father a larger amount of normal chromatin than do the male offspring. So that the female arises from an egg in which equal amounts of good and injured chromatin are present, while the male offspring arises from an egg in which a larger amount of injured chromatin is united with a smaller amount of normal. Therefore, proportionately, the male offspring from treated mothers have more injured chromatin in their entire bodily make-up than do the female offspring, and are comparatively in a more abnormal condition. This reasoning refers only to the immediate offspring of alcoholic parents and not to their later descendants.

Another explanation of these differences between the male and female offspring of alcoholized females could be based on the possibility of the female being heterozygous for sex. This involves a very complex discussion, but one for which there is some ground on the basis of the regulation of the sex ratio in these animals.

Finally, then, the experiments show the hereditary transmission through several generations of conditions resulting from an artificially induced change in the germ cells of one generation by treatment with alcohol. And they furnish data of importance bearing upon the pathological behavior of the carriers of heredity as well as the differences in behavior between the two types of germ cells produced by an animal carrying heteromorphic chromosomes.

It is impossible in an article of this length to give much of the important detail of the experiments, such as the records of successive pregnancies of normal females by normal and alcoholic mates and many other individual responses which are necessary for a full understanding of the conditions and results. Should the reader be further interested in the biological aspects of this problem he may refer to the publications cited below in which the author has recorded in a more detailed manner experimental studies conducted for the past seven years in analyzing the effects of alcohol on the development of various classes of vertebrates.

BIBLIOGRAPHY.

Stockard: The Influence of Alcohol and Other Anesthetics on Embryonic Development. (*Amer. Jour. Anat.*, 1910, Vol. 10, p. 369.)

An Experimental Study of Racial Degeneration in Mammals Treated with Alcohol. (*Archiv. Int. Med.*, 1912, Vol. X.)

The Artificial Production of Eye Abnormalities in the Chick Embryo. (*Anat. Record*, 1914, Vol. 8, p. 33.)

Stockard and Papanicolaou: A Further Analysis of the Hereditary Transmission of Degeneracy and Deformities by the Descendants of Alcoholized Mammals. (*Amer. Naturalist*, Parts I and II, Vol. L, February and March, 1916, p. 65.)

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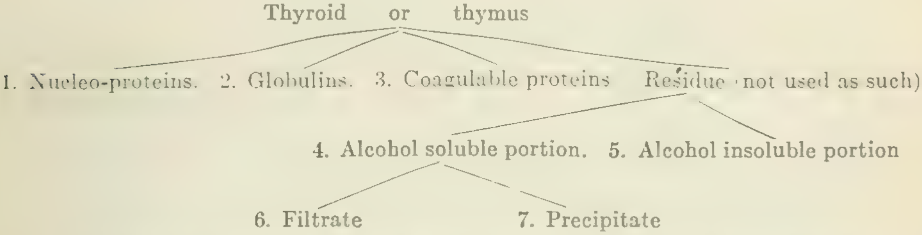
26. *Studies on internal secretion IV. Treatment of tadpoles with thyroid and thymus extracts.* J. F. Gudernatsch, Department of Anatomy, Cornell University Medical College, New York City.

In some previous experiments on growth and differentiation portion of fresh thyroid, thymus and other glands were given as food to developing tadpoles. At the same time the animals lived in aqueous extracts of the entire glands, some of the tissue juices, of course, going into solution. In the experiments reported here an attempt was made to study the influence upon the development of tad-poles of several distinct constituents of the thyroid and thymus. By a detailed chemical procedure each organ was split into seven products, all in aqueous solutions.[1]

Six of these portions contained each one 'agent,' the seventh, termed the alcohol soluble portion, combined two 'agents,' precipitate and filtrate, before the process of precipitation.

The seven isolated portions were:



The nitrogen content of the solutions was determined and the same concentration used for each set of tadpoles. An ordinary meat and vegetal diet was given.

The thyroid and several of its constituents proved the most powerful stimulants for accelerated differentiation, while growth was suppressed almost entirely. When graded according to their action on differentiation the thyroid portions give the following list:

(Thyroid, entire gland, dessicated; Parke, Davis and Company)
Thyroid nucleo-proteins.....................................no growth
Thyroid globulins
Thyroid acohol insoluble portion
Thyroid coagulable proteins
Thyroid alcohol soluble portion
Thyroid filtrate
Thyroid precipitate.................................greatest growth
Control

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[1] The solutions were prepared by the Department of Experimental Medicine.

The last three products hardly showed any effect on differentiation, the tadpoles treated with the precipitate running almost parallel to the control. Tadpoles treated with the first four portions, however, metamorphosed from five to six weeks before the control, while they never reached the size of the control animals. Nucleo-proteins and globulins checked growth entirely.

Three sets of the experiment started at different ages of the tadpoles —each interval being about four weeks—showed strikingly that older animals react to the thyroid treatment faster than younger, being, of course, nearer the stage of metamorphosis. In the youngest set the nucleo-proteins required twenty days to bring about metamorphosis, in the next set six days and in the oldest four days.

It has been claimed that the amount of iodine present in the thyroid is mainly responsible for its action. It might be possible to determine this question by using the several thyroid products in the same iodine concentration. In the present experiment they were equalized as to their nitrogen content. Graded in regard to the iodine present they range as follows:

Nucleo-proteins
Coagulable proteins and alcohol soluble portion
Globulins
Alcohol insoluble portion
Filtrate
Precipitate

This column does not run parallel with the previous one, although the nucleo-proteins and the precipitate occupy the corresponding places.

Considering the action of the thymus as a whole, viz., retardation of differentiation, these experiments furnished some bewildering results. Only two of the seven products of the thymus delayed differentiation considerably, the alcohol soluble portion and the precipitate. The rest brought about differentiation simultaneously with or ahead of the control. It is impossible at present to explain these facts. It becomes necessary to assume either that the thymus furnishes several products of different physiological properties or that five of the seven constituents of the fresh gland are inactive. The latter assumption does not seem plausible; for the mass of the nucleo-proteins alone, for instance, is far greater than the alcohol soluble portion.

Beginning with that thymus product most active in retardation a graded list of the seven constituents would read as follows:

Thymus precipitate
Thymus alcohol soluble portion........................slowest growth
(Thymus entire gland, dessicated; Parke, Davis and Company)
Thymus filtrate
Control
Thymus alcohol insoluble portion
Thymus globulins
Thymus nucleo-proteins..................................fastest growth

The expectation that the corresponding constituents of the two glands would be most active in counteracting each other was not realized; for it will at once be seen that in general the serial arrangement of the thymus products is almost the opposite of that of the thyroid series.

It is impossible here to give all the growth curves. In the thyroid group the nucleo-proteins (strongest differentiator) permitted no growth, the filtrate and precipitate (weakest differentiators) allowed the tadpoles to grow to normal size in about the normal time. Thyroid filtrate and precipitate may well be considered inactive as far as differentiation is concerned. They may have other physiological properties not disclosed in these experiments. In fact, injection experiments on mammals revealed such properties, especially in the case of the filtrate. In the thymus group the nucleo-proteins gave most, the alcohol soluble portion least rapid growth. Both have their position at opposite ends of the above list showing their influence on differentiation.

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November, 1916

ON THE DEVELOPMENT OF THE ATRIAL SEPTUM AND THE VALVULAR APPARATUS IN THE RIGHT ATRIUM OF THE PIG EMBRYO, WITH A NOTE ON THE FENESTRATION OF THE ANTERIOR CARDINAL VEINS

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NINE FIGURES

The method of formation of the atrial septum and nearly related parts in the mammalian heart has been known since the careful investigations of Röse ('88 and '89), Born ('89), and more recently Favaro ('13). These writers are in substantial agreement regarding the structures concerned in this process, though differing in some respect as to detail. The now classical work of Born on the rabbit has been used extensively as a basis for descriptions of cardiac development in some of the more recent text-books of embryology, notably by Hochstetter ('01–'03) and Tandler ('12 and '13). Born in his account, which included some observations on human embryos, corrected the error of His ('85) regarding the formation of the atrial septum and its relation to the foramen ovale. His description differed from the earlier one of Röse ('88) in several important respects but later Röse ('89) accepted Born's corrections. Röse and Favaro extended their observations to a number of different mammals, but not, as far as I am aware, to the pig. Retzer ('08) published a brief note on the development of the heart in which he claimed that Born's account of the atrial septum in the rabbit could not be applied to the pig. Since pig embryos are extensively used for study in American laboratories, it seemed advisable to re-examine the development of the septal and valvular apparatus in this form in order to clear up any uncertainty which may exist regarding the processes involved.[1]

This study is based on series of pig embryos of 6.8, 7.9, 8.5, 12.3, 15.2 and 21 mm. total lengths, supplemented by dissections of the hearts of foetuses from the 45 mm. stage to birth. The hearts of the 7.9, 15.2 and 21 mm. embryos were reconstructed by the wax-plate method. In addition several human embryos from 7.6 to 22 mm. were examined for comparison.

The terminology of Born which has been largely adopted by other writers will be used, with some modifications, in this account.

In the stage represented by a 6.8 mm. embryo, septum I forms an incomplete interatrial curtain. Ostium I is still widely open and ostium II has begun to form. In the reconstruction of a 7.9 mm. embryo (fig. 5), the relation of these three structures is shown. Septum I (*S.I*) though fused for the most part with the atrial walls, still presents a short, free border facing the now very narrow ostium I (*O.I*). In the posterior superior corner of the septum, the new opening or ostium II (*O.II*) is well advanced and much larger at this time than ostium I. It is apparent from the figure that ostium II is formed by fenestration in the pig and is not at first a single opening as Born found in the rabbit. Born thought that a primitively single opening was normal for mammals generally, but Röse found a fenestrated septum in three cow embryos, a mole embryo and two rabbit embryos and states that Bruch's observations on sheep, cow and horse and Rokitansky's[2] on a human embryo support the conclusion that fenestration is the more usual condition. Favaro found numerous orifices, representing ostium II in the sheep, but only a single opening in the guinea-pig. As a rule, Röse believes, the larger openings coalesce to form a single ostium II while the smaller ones close up.

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[1] This investigation was begun in the anatomical department of the University and Bellevue Hospital Medical College and completed at Cornell University Medical College, New York.

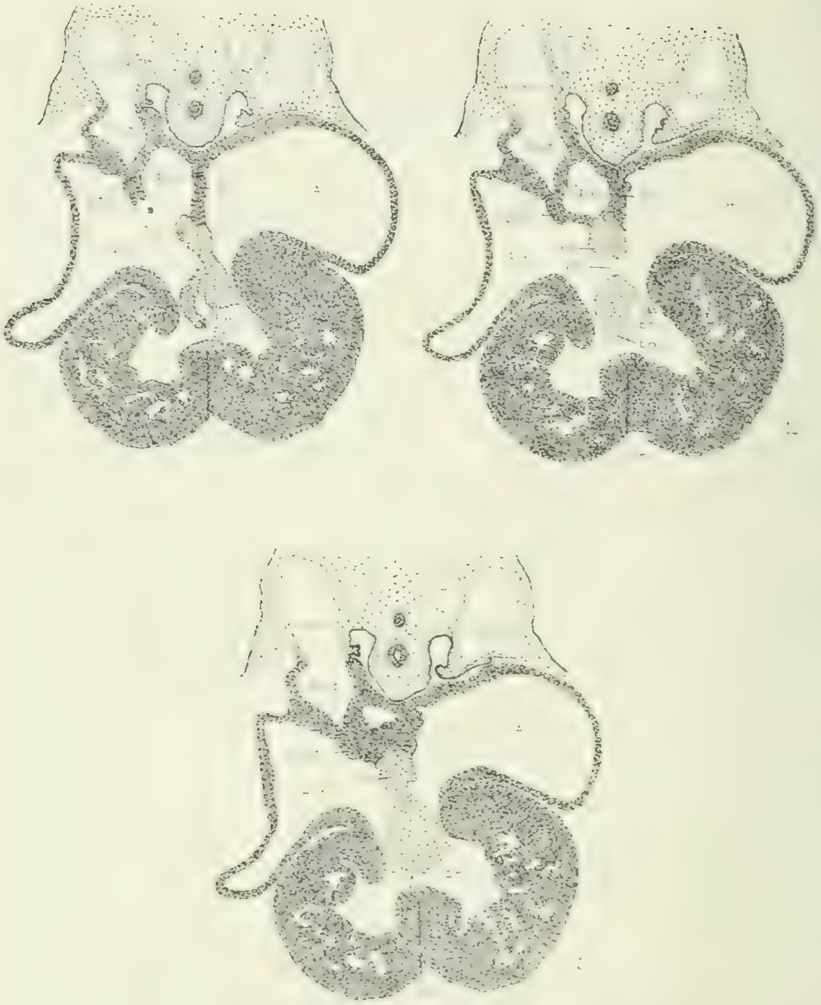
[2] The observations of Bruch and Rokitansky are known to me only through the brief mention made by Röse ('88).

Exceptionally the latter may persist until birth. In the pig later stages always show a single opening.

The closure of ostium I in the pig is effected in the same manner as described for the rabbit, guinea-pig, man and other mammals. This process is illustrated in figures 1, 2, 3, and 5. Septum I is composed for the most part of developing muscle which is continuous with the musculature of the roof, posterior wall and floor of the atrium. Its free border, however, is capped by a prominent endocardial thickening. As the septum grows downward and forward toward the atrial canal, the endocardial thickening develops two extensions or horns which fuse with the endocardial cushions of the atrial canal. The upper one blends with the upper cushion (fig. 1), the lower one with the lower cushion (fig. 3). Ostium I (fig. 2) is thus entirely surrounded by thickened endocardium except where it is continuous with the narrow slit (transverse fissure) between the endocardial cushions of the atrial canal. At a slightly later stage (8.5 mm.) the blending of the separate portions of endocardium is complete and ostium I together with the transverse fissure is closed. In the 15.2 mm. stage (fig. 6) ostium II (*O.II*) is much enlarged and forms a single large opening between the atria. Septum I (*S.I*) presents a very irregular free border which faces upward and slightly posteriorly toward ostium II. (The irregularity of the free border could not be represented in wax.) The septum as a whole bends toward the cavity of the left atrium.

The mode of formation of septum II (the later limbus Vieussenii) is first clearly indicated in the 7.9 mm. embryo though a faint trace is sometimes distinguishable in an earlier stage. Concerning the origin of this septum there has been some difference of opinion. Indeed Retzer ('08) has denied its existence in the pig, though Röse ('88 and '89), Born ('89) and Favaro ('13) have found it in all the forms they studied, Röse expressly stating that it is characteristic of all placental mammals. In the present study it will be shown that septum II is as definite and well developed in the pig as in any form so far examined.

The model of the 7.9 mm. embryo (fig. 5) shows a very distinct spur-like thickening (*S.II*) which projects into the cavity



Figs. 1 to 3 Transverse sections of the heart region of a 7.9 mm. embryo (histological structure semi-diagrammatic). Figures 1 and 3, above and below the level of ostium I respectively; figure 2, through ostium I. *A.*, dorsal aorta; *A.d.*, right atrium; *A.s.*, left atrium; *En.s.*, upper endocardial cushion; *En.i.*, lower endocardial cushion; *O.I*, ostium I; *S.in.*, interventricular septum; *S.I.*, septum I; *S.II*, septum II; *Sn.d.*, right sinus horn; *Sp.in.*, spatium interseptovalvulare; *V.car.c.d.*, and *V.car.c.s.*, right and left common cardinal veins; *V.v.d.*, right sinus valve; *Vv.v.*, sinus valves. Magnified about 25 diameters.

of the right atrium in the angle between the floor and septum I, and just posterior to ostium I (*O.I*). It is continuous with the left sinus valve. Transverse sections through this region (figs. 1, 2 and 3) show that at first this spur (*S.II*) is composed of connective tissue alone and is sharply marked off, histologically, from the developing musculature of the sinus valves, septum I and the atrial floor. It appears to be nothing more than a projection, toward the right, of the endocardial thickening of septum I (*S.I*) and is continuous through the latter with the endocardial cushions of the atrial canal (*En.s.* and *En.i.*). This structure which is the anlage of septum II, is undoubtedly what His ('85) called the spina vestibuli and, taken together with the endocardial cushions, would constitute his septum intermedium. The lower end of the left sinus valve blends with it, while the corresponding end of the right valve flattens down in the atrial floor close to it (figs. 2 and 5, *V.v.*). At this stage, the anlage of septum II is not in relation with ostium II.

In later stages, when ostium I has completely closed, the spur lengthens out into a definite ridge. This is well shown in the model of a 15.2 mm. embryo (fig. 6, *S.II*). When traced from its point of origin in the anterior inferior corner of the atrium, this ridge extends upwards then bends backward along the roof toward the posterior wall where it flattens down and may become continuous with two or three muscular trabeculae which develop in this region, i.e., the spatium intersepto-valvulare. These trabeculae are not constant in number. The portion of the ridge in relation to the roof of the atrium[3] is broad and low contrasted with its lower anterior end or root which is sharp and well-defined. Both sinus valves (*V.v.s.* and *V.v.d.*) now blend with it, the right, however, slightly covering its lateral side.

The internal structure of septum II is shown in a transverse section taken at about the middle of its anterior vertical portion (fig. 4, *S.II*). The endocardial thickenings which entered into its formation in an earlier stage, have fused together into a solid quadrilateral mass. A cap of developing muscle covers its posterior border (upper in the figure) and extends anteriorly for some distance along its lateral surfaces. This muscular mass is continuous with the musculature of septum I and, at a lower level (not shown in the figure), with that of the two sinus valves which, as mentioned, blend with the root of the septum. A careful study of serial transverse sections shows that the musculature of this part of septum II is derived from that of

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[3] Owing to the position of the model this part of septum II is hidden in figure 6.

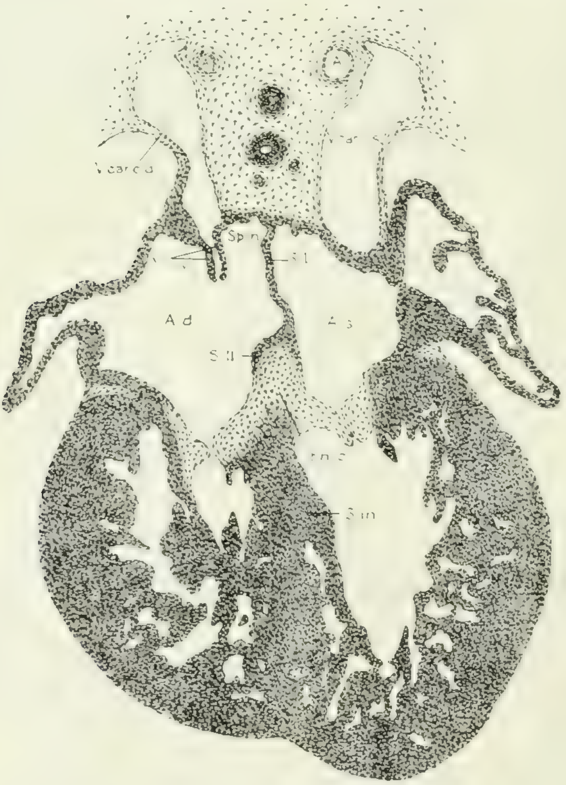

Fig. 4 Transverse section of the heart region of a 15.2 mm. embryo at the level of the root of septum II (histological structure semi-diagrammatic). *A.*, dorsal aorta; *A.d.*, right atrium; *A.s.*, left atrium; *En.c.*, fused endocardial cushions; *S.in.*, interventricular septum; *Sp.in.*, spatium intersepto-valvulare; *S.I.*, septum I; *S.II*, septum II; *V.car.c.d.*, and *V.car.c.s.*, right and left common cardinal veins. Magnified 25 diameters.

the sinus valves and septum I. The developing muscle elements appear to invade the connective tissue and gradually to replace it.[4] This invasion has not yet begun in the 8.5 mm. embryo but is well advanced in the 12.3 mm. and still more so in the 15.2 mm. described above. The anterior portion of the connective tissue mass or septum intermedium[5] (fig. 4 *En.c.*) broadens out in a lateral direction between the two atrio-ventricular orifices and is joined by the interventricular septum, the line of fusion being toward the right.[6] The fusion is not complete, however for the interventricular ostium (fig. 6 *O.in.*) is still widely open at this stage. If septum II is now traced upward and backward into the roof of the atrium, the connective tissue elements gradually disappear and it appears as a low, broad thickening of the atrial musculature which follows the external depression caused by the bulbus in this region. It is not, however, due to an infolding of the atrial wall, but to a local increase in the developing muscle elements. This upper part of septum II now comes into relation with ostium II (fig. 6, *O.II*) which meanwhile has become much larger.

Passing now to the 21 mm. embryo, the further development of septum II (fig. 7, *S.II*) is clearly shown. It appears as a crescentic ridge extending from the lower inferior corner of the atrium, upward and backward toward the posterior wall. Its lower segment or root which is thickest and most sharply defined is partly hidden by the high right sinus valve (*V.v.d.*) which blends with its lateral surface. Traced upward it curves over the roof of the atrium bordering ostium II (*O.II*) and reaching the posterior wall, bends downward somewhat in the region of the spatium intersepto-valvulare (not shown in the

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[4] The invasion of the connective tissue mass (septum intermedium) by the musculature of the sinus venosus was described by Retzer ('08) as the source of the Purkinje fibres in the ventricles. The present investigation leads to the conclusion that a part of this muscle, at least, thickens to form the root of septum II.

[5] The term 'septum intermedium' originally used by His ('85), may be conveniently retained for this structure as suggested by Favaro ('13), although Born and some others discarded it.

[6] A portion of the septum intermedium thus separates the right atrium from the left ventricle (conus arteriosus of the aorta) forming the septum atrioventriculare of Hochstetter.

figure). This upper segment is broad and low where it faces ostium II but becomes narrow and sharp in the posterior wall. Septum I meanwhile has changed its position relative to septum II, so that its free border now faces upward and forward, and ostium II has become an oblique cleft, the definitive foramen ovale, between the septa.

Before considering the later changes in septum II it will be convenient to compare the conditions found in the pig with those described for other forms. According to Born septum II first appears (rabbit and man) in the upper part of the posterior wall of right atrium a little to the right of septum I. It is a crescentic spur which encroaches upon the spatium interseptale and forms the principal part of the limbus Vieussenii. In the pig irregular muscular ridges develop in the same locality but they vary greatly in form, size and number. In the model of the 15.2 mm. embryo, two such ridges can be distinguished (fig. 6). When septum II has extended backward to the posterior wall, one or more of these ridges is incorporated in it, but no one of them is sufficiently well-marked to be taken as a starting point for the developing septum. Further, at the time when the anlage of septum II is first distinguishable in the lower anterior wall (7.9 mm. embryo) the spatium intersepto-valvulare is relatively very wide and numerous small irregular ridges appear in its upper posterior wall. Along with the narrowing of the spatium, the ridges decrease in number until finally they are reduced to one or two which, as stated, are incorporated in the septum.

An examination of several human embryos seemed to indicate that much the same sort of process takes place there. In an embryo of about 12 mm., total length, at least three muscular ridges could be distinguished in the upper, posterior wall of the spatium intersepto-valvulare, while in one of about 14 mm. only one such ridge appeared which, however, was quite prominent.[7] In the lower anterior part of the right atrium of both these embryos there was a thick connective tissue ridge or prominence continuous with the fused endocardial cushions and partly overlaid and invaded by developing muscle. As in the pig this musculature was continuous with that of septum I and the sinus valves. The resemblance between this structure and that described for the pig in the corresponding place is so close that I am inclined to think it represents the origin of septum II in man. Indeed Thyng ('14) in his description of a human embryo notes the presence of a "ridge or tubercle" in the same region and states that "from the relation which the tubercle bears to the septum primum and the left sinus valve it can scarcely be doubted that it would eventually form part of the adult limbus fossae ovalis."

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[7] Through the kindness of Dr. Thyng I have had the opportunity of examining the human embryo which formed the subject of his paper ('14). In this specimen (17.8 mm.), the region of the spatium intersepto-valvulare was entirely devoid of muscular ridges.

Born seems to have overlooked the early appearnace of this ridge entirely, though he states that in man the bay of the crescent (septum II) swings further downward in later stages and finally unites with the lower end of the left sinus valve. He thus includes the region in which the ridge is found in the pig. In the rabbit, however, septum II remains as a narrow short crescent in the anterior upper wall of the atrium. One may conclude from this that the ridge if present at all is poorly developed in this animal.

Röse ('89) in summarizing the results of his earlier work ('88) speaks of septum II (limbus or annulus ovalis) as follows:

> On the anterior and upper atrial wall there appears a ridge-like infolding, the septum musculare which, together with the septum intermedium . . . . . is formed into a closed ring diaphragm, the annulus ovalis . . . . . The septum intermedium arises from a connective tissue spina vestibuli overlaid, however, on its upper surface by a thin continuous muscle layer, which unites with the broad atrioventricular cushions, the latter fused together in the middle.

It is apparent that this description corresponds very closely with that given for septum II in the pig with the exception that the upper part of this septum corresponding to Röse's septum musculare is not formed by an infolding of the atrial wall but by a local thickening. In his later paper ('89) following Born's work, Röse modified his description of the atrial septa extensively. He rejected the idea that the septum intermedium is concerned in the formation of the limbus or annulus ovalis. He also discarded the term 'septum musculare,' calling it simply the limbus (Vieussenii) and described it as arising in the anterior upper atrial wall as an infolding caused by the truncus arteriosus (bulbus) imbedding itself between the two atria. From here it spreads along the upper and then along the lower wall of the right atrium. It contains a connective tissue core which was pinched off from the surface of the truncus during the process of infolding. The presence of this core, Röse thinks, is a proof that infolding has occurred.

In the pig the upper part of septum II or limbus which corresponds to the curve of the bulbus, is at first a broad thickening of the musculature of the atrial wall which in later stages becomes more prominent. Only in its lower anterior part or root are connective tissue elements found. These are the remains of the original thickened endocardium which was shown to form the basis for the septum. Retzer ('08) in denying the presence of a septum II in the pig says: "This supposed septum which His correctly described as a 'muskulöse Leiste' is nothing but a fold in the atrial wall at that place." It is formed by the atrial growing around the conus arteriosus as a fixed point, thus causing a bulging inwards of the atrial wall.

The descriptions of Röse and Retzer thus correspond quite closely as far as the origin of the muscular fold is concerned. Retzer, however, thinks it never attains sufficient size to be called a septum. His ('85) described the same structure, calling it the anterior septum or 'sickle' which later gives rise to the limbus Vieussenii. He thought that it was formed by the septum intermedium growing up on the anterior atrial wall to meet the anterior end of the septum superius (septum I). This upgrowth of the septum intermedium, or a portion of it overlaid by muscle, is practically what takes place in the pig, but as pointed out previously, there is no evidence of an infolding of the atrial wall at a higher level.

Favaro's ('13) recent account of the septum secundum in the guinea-pig and sheep differs very little from that given above for the pig. He finds that the myocardium which invades the connective tissue of the septum intermedium becomes more compact and raised up into a distinct prominence on the right side of the insertion of the atrial septum (septum I). This, he says, represents the site of the inferior segment of the limbus fossae ovalis. The prominence is continued upward as a thickening of the myocardium of the ventral wall of the atrium on the right of septum I. It corresponds to a broad sulcus externally but there is no infolding as described by Röse and Retzer. In the guinea-pig and sheep, however, Favaro finds that the septum spurium or tensor valvulae bends over the upper wall to join the newly-found limbus or septum II. This does not occur in the pig as reference to figures 5, 6 and 7 will show. In this form the tensor valvulae[8] (*T.vv.*) extends upward to the roof of the atrium where it is lost sight of among the developing trabeculae in that region. In the human embryos examined the course and relations of the tensor seemed to be more like those in the guinea-pig and sheep as described by Favaro.

From the first the lower end of the left sinus valve blends with septum II as mentioned previously. The right valve is at first independent of the septum (fig. 5) but later when the septum has increased in height, the valve becomes attached to its right side (figs. 6 and 7). In later stages the right valve increases rapidly in height so that when seen from the right side, it completely covers the lower end of the left valve and the root of septum II (fig. 7). When a portion of the right valve is cut away (fig. 8) the connection between the left valve and septum II can be seen. Separating the orifices of the inferior vena cava (*V.c.i*) and the coronary sinus[9] (*Sn.cor.*) is a thick ridge, the sinus septum (*S.sn.*), which extends from the root of septum II downward and to the right toward the attached border of the right sinus valve, where it blends with the floor of atrium. Owing to the extreme height of the right valve the inferior vena cava and coronary sinus appear to open into a common chamber flanked by the valve.

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[8] The term 'tensor valvulae,' first proposed by Röse ('89), seems more appropriate than the older 'septum spurium' of His. This structure probably helps to approximate the valves and render them tense during systole.

[9] When the model was sawn through, the cut opened the right extremity of the coronary sinus which forms a deep bay as it turns backward and upward to its orifice (figs. 7 and 8).

The further changes in the right atrium may now be considered. From the 21 mm. stage (figs. 7 and 8) up to about 58 mm. the relations of the parts in question change very little. Septum I or the valvula foraminis ovalis, as it may now be called, becomes thinner and somewhat folded. Its free border is fimbriated. The left sinus valve is still well developed, its lower end blending with the root of septum II. It now lies so close to the septal wall of the atrium that the spatium interseptovalvulare is reduced to a narrow cleft. The right sinus valve remains high, guarding the orifices of the inferior cava and coronary sinus. The sinus septum between the two orifices is behind and medial to the valve but not fused with it. In two specimens (58 to 62 mm.) and possibly a third, a new opening appeared in the lower anterior part of the right sinus valve close to its attached border. The course of the blood-stream from the coronary sinus in this case was directly into the lower anterior part of the atrial cavity rather than upward, backward and medially toward the foramen ovale. The usual pathway, however, was not shut off. His ('85) described a secondary opening of the coronary sinus in human embryos and assumed that it represented the normal condition. Other writers have not found this new opening, Born expressly denying its existence. It probably occurs exceptionally in man as in the pig and may represent a premature degeneration of this part of the valve.

The tensor valvulae (septum spurium) in the older foetuses becomes flattened down considerably in its upper part, though still fairly well marked near the sinus valves. Meanwhile a new muscular ridge, the crista terminalis has appeared in the atrial wall just to the right of the right valve. Its upper part blends with the trabeculae in the roof of the atrium near the tensor valvulae. Traced downward it passes first close to the attached border of the valve and then diverges from it more and more until it is lost among the trabeculae of the lateral wall of the atrium. The right sinus valve does not seem to take any part in its formation, though both Röse and Tandler ('13) consider that the upper part of this valve is involved.

The final disposition of the various parts of the septal and valvular apparatus is shown in figure 9 which represents a dissection of the heart of a foetus of about 85 mm. Owing to the preponderating growth of the upper part of the atria, the region of the septa appears sunken into the entrance of the inferior cava, while the orifice of the superior cava (*V.c.s.*) remains high up on the posterior wall. Septum I, now the valvula foraminis ovalis (*V.f.o.*) lies in the bottom of a slight depression or fossa whose margin is formed for the most part by the limbus fossae ovalis (*L.V.*). The free, fimbriated border of the valvula extends obliquely into the cavity of the left atrium and overlaps the limbus on that side. The oblique cleft between them is the foramen ovale. Above the fossa and extending downward on each side of it, the limbus is thick and muscular. This part is formed by septum II.

Continuous with the anterior end of septum II is the left sinus valve (*V.v.s.*) which in this place is muscular like the septum and may be said to form part of the limbus. When traced backward and upward around the fossa, the valve becomes reduced to a thin, pale streak which finally crosses the posterior part of the limbus proper and then extends along the left margin of superior caval orifice where it gradually fades out. This is practically in agreement with the descriptions given by Born, Röse and others.

The right sinus valve (*V.v.d.*) is still well marked at this stage. Its lower anterior part bounds the orifice of the inferior cava laterally and extending forward and medially partially covers the orifice of the coronary sinus. The first portion represents the valvula Eustachii, the last (*X* in fig. 9) the valvula Thebesii of other mammals. In the pig the right sinus valve never becomes divided structurally. There is always a narrow cleft between it and the sinus septum (*S.su.*). The upper part of the right valve extends as a thin narrow membrane along the right border of the superior caval orifice almost to its upper end. The tensor valvulae has entirely disappeared.

At this stage the crista terminalis (*Cr.ter.*) forms a thick ridge extending downward from the roof, in the angle between the posterior and lateral walls of the atrium. It was pointed out that when the crista first appears it is entirely independent of the right sinus valve, but as it becomes broader and more fully developed, the extreme upper part of the valve comes to lie right on its root. There is usually, however, a narrow cleft separating it from the remains of the valve below. For this reason, the crista terminalis of the pig cannot be said to mark exactly the boundary between the primitive sinus cavity and the atrium proper as has been maintained by His, Röse and Tandler for man.

A glance at figure 9 will show that there is a broad, rounded ridge in the angle between the orifices of the superior and inferior venae cavae (the line marked *V.v.s.* passes across it). It is formed by a thickening of the musculature of the atrial wall in this region and represents the tuberculum intervenosum (Loweri) of the human heart. There has been some doubt about the occurrence of this structure but recently Tandler ('13) came to the conclusion that it is always present in the adult human heart if hardened in situ, i.e., with the pericardium intact. He suggests the term 'torus Loweri' as more appropriate. In other mammals he finds it more strongly developed than in man, especially in the horse and still more so in the seal where it forms a 'veritable septum.' In the pig this ridge occurs constantly in older foetal stages. Owing to its position it would tend to direct the blood-stream from the superior cava toward the right atrio-ventricular orifice.

The chief results of this investigation may be summarized briefly as follows:

In the pig, the method of formation of septum I and ostia I and II is essentially the same as described in other mammals.

The present account of septum II, however, differs considerably from those of other writers with the exception of Favaro. The anlage of this septum in the pig appears in the lower anterior corner of the right atrium. It is a spur-like thickening developed on the right side of the line of insertion of septum I and is composed, at first, of connective tissue. Later it lengthens out and becomes overlaid and invaded by developing muscle. It then extends upward and backward along the roof of the atrium as a low, broad thickening of the musculature in this region and finally reaches the posterior wall where it bends downward as a narrow, sharp ridge. For the reasons stated in the text, I am inclined to believe that septum II is formed in the same manner in the human embryo.

The right sinus valve is well-developed in the pig. Its lower part guards the orifices of the inferior vena cava and coronary sinus in later stages but does not become divided by the sinus septum into Eustachian and Thebesian valves as in other forms.

The crista terminalis develops independently of the tensor valvulae (septum spurium) and right sinus valve. It does not accurately mark off the primitive sinus cavity from the atrium proper.

A tuberculum or torus Loweri is always present in older foetal stages.

One further point which is illustrated by the models may be mentioned although it is not directly related to the subject of this paper, namely, the fenestration of the proximal part of the anterior cardinal vein. In the 7.9 mm. embryo (fig. 5, *V.car.a.*) this process is well advanced and many of the fenestrae have coalesced, thus tending to separate off a dorsal portion of the cardinal which would receive the intersegmental veins in this region. In the 15.2 mm. embryo (fig. 6, *V.car.a.*) the separation is complete up to the point where the subclavian vein (*V.scl.*) enters. On the left side (not shown in the figure) where the subclavian appears to enter one segment higher (cephalad) than on the right, the separation is carried upward correspondingly. These observations support the opinion of Thyng ('14) that the proximal part of the vertebral vein is segregated from the main venous channel (anterior cardinal) in the manner described. In an earlier paper Thyng ('11) figured this condition in a pig embryo of 7.8 mm. Lewis ('03) mentions a splitting of the anterior cardinal vein near its entrance into the duct of Cuvier, the subclavian vein arising from its outer part. A comparison of his Plate IV and Thyng's ('11) figure 2, with figures 5 and 6 of the present paper seems to indicate that this process is normal for the pig embryo.

In conclusion I wish to express my thanks to Prof. H. D. Senior who kindly suggested a re-investigation of the atrial septum and to Prof. F. W. Thyng for advice and criticism.

LITERATURE

Born, G. 1888 Über die Bildung der Klappen, Ostien und Scheidewände in Säugerthierherzens. Anat. Anz., Bd. 3.

1889 Beiträge zur Entwickelungsgeschichte des Saugerthierherzens. Arch. f. mikr. Anat., Bd. 33.

Favaro, G. 1913 Ricerche embriolog. ed. anatom. intorno al cuore dei vertebrati, Parte I. Padova.

His, W. 1885 Anatomie menschlicher Embryonen. III. Zur Geschichte der Organe. Leipzig.

Hochstetter, F. 1901–1903 Die Entwickelung des Blutgefässsystems. Hertwig's Handbuch der vergleich. und experim. Entwickelungslehre der Wirbeltiere. Bd. 3, 1906.

Lewis, F. T. 1903 The gross anatomy of a 12-mm. pig. Am. Jour. Anat., vol. 3.

Morrill, C. V. 1915 A preliminary note on the septum secundum in the pig. Anat. Rec., vol. 9, p. 111.

Retzer, R. 1908 Some results of recent investigations on the mammalian heart. Anat. Rec., vol. 2, p. 149.

Röse, C. 1888 Beiträge zur Entwicklungsgeschichte des Herzens. Dissert. inaug. Heidelberg.

1889 Zur Entwicklungsgeschichte des Säugerthierherzens Morph. Jahrb., Bd. 15.

1890 Zur Vergleichenden Anatomie des Herzens der Wirbelthiere. Morph. Jahrb., Bd. 16.

Tandler, J. 1912 The development of the heart. Keibel and Mall's Manual of Human Embryology.

1913 Anatomie des Herzens. Bardeleben's Handbuch der Anatomie des Menschen Bd. 3, Abt. 1.

Thyng, F. W. 1911 The antomy of a 7.8 mm. pig embryo. Anat. Rec., vol. 5.

1914 The anatomy of a 17.8 mm. human embryo. Am. Jour. Anat., vol. 17.

PLATE I

EXPLANATION OF FIGURES

5 Reconstruction of the heart of a 7.9 mm. embryo. The right atrium and right ventricle have been opened from the right side, the cut passing through the junction of the transverse part of the sinus venosus (*Sn.t.*) and the right sinus horn (*Sn.d.*). Magnified 25 diameters.

6 Reconstruction of the heart of a 15.2 mm. embryo, the right atrium and right ventricle opened from the right side. Magnified 25 diameters.

ABBREVIATIONS

A., Aorta dorsalis
Ao., Aorta (ventral part)
A.d., Atrium dextrum
A.s., Atrium sinistrum
Bulb., Bulbus cordis
Cr.ter., Crista terminalis
En.s., upper endocardial cushion
En.i., lower endocardial cushion
En.c., fused endocardial cushions
L.V., Limbus fossae ovalis (Vieussenii)
O.I, Ostium primum
O.II, Ostium secundum
O.in., Ostium interventriculare
Per., Pericardium
S.I, Septum primum
S.II, Septum secundum
S.in., Septum interventriculare
Sn.t., Sinus venosus, trans. part
Sn.d., Sinus venosus, right horn
S.sn., sinus septum
Sn.cor., Sinus coronarius
Sp.in., Spatium intersepto-valvulare
Sul.in., Sulcus interventricularis
T.vv., Tensor valvulae = Septum spurium
Vv.v., Valvulae venosae
V.v.d., right valve
V.v.s., left valve
V.f.o., Valvula foram. ovalis
V.car.c.d., right com. card. vein
V.car.c.s., left com. card. vein
V.car.a.d., right ant. card. vein
V.car.p.d., right post. card. vein
V.c.s., Vena cava superior
V.c.i., Vena cava inferior
V.az., Vena azygos
V.scl., Vena subclavia
V.h., Vena hepatica
X, (fig. 9) = part of right sinus valve guarding coronary orifice

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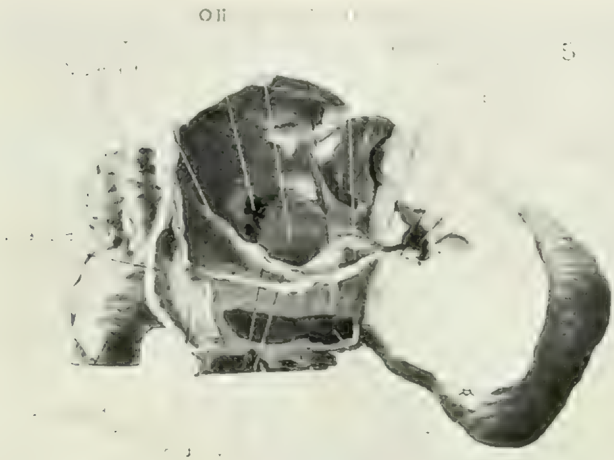

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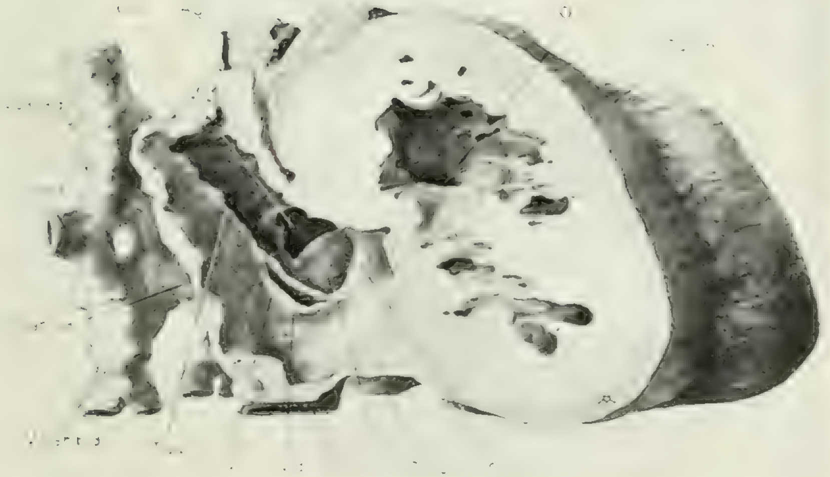

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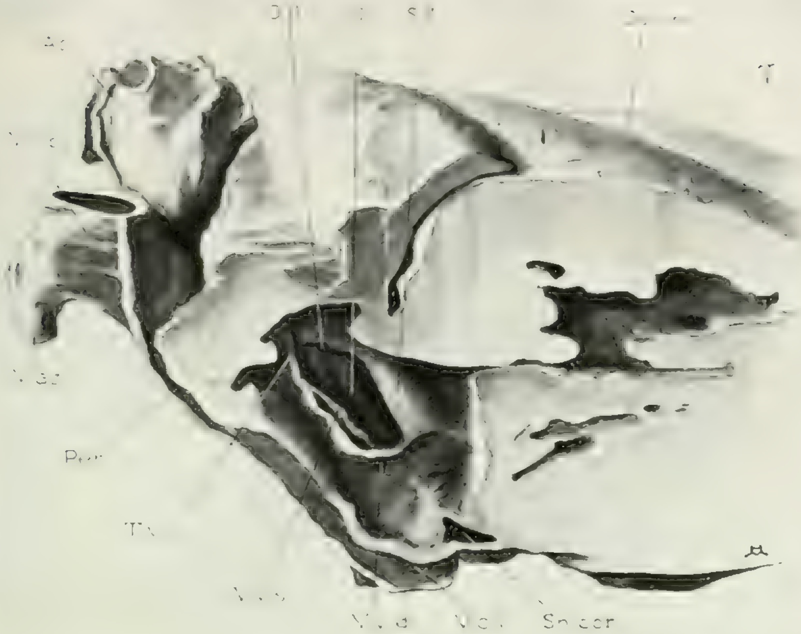
PLATE 2

EXPLANATION OF FIGURES

7 Reconstruction of the heart of a 21.0 mm. embryo. The right atrium and right ventricle have been opened from the right side, the cut passing through a part of the coronary sinus (*Sn.cor.*). Magnified about 25 diameters.

8 Same as figure 7 but in slightly different position. The right sinus valve (*V.v.d.*) has been partly cut away, exposing the orifices of the coronary sinus and inferior vena cava. Probes have been inserted in the superior and inferior cavae and the coronary sinus. The probe in the coronary sinus passes across the sinus septum (*S.sn.*) under the right valve. Magnified about 25 diameters.



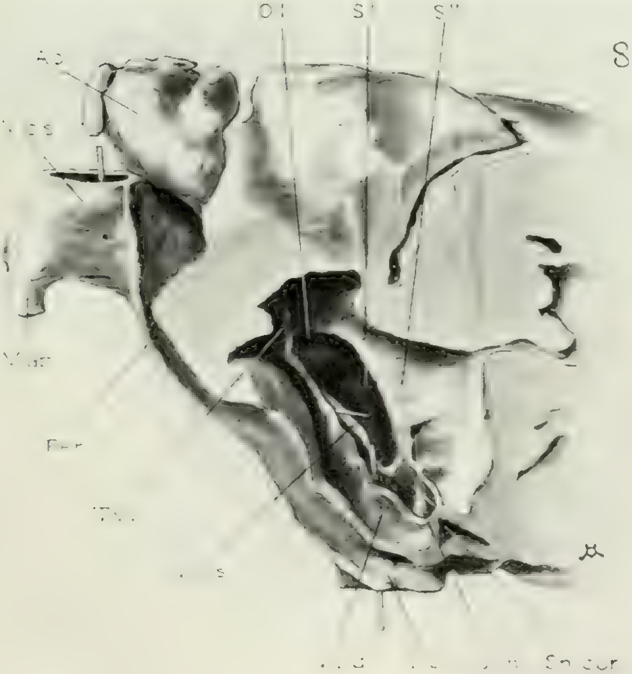


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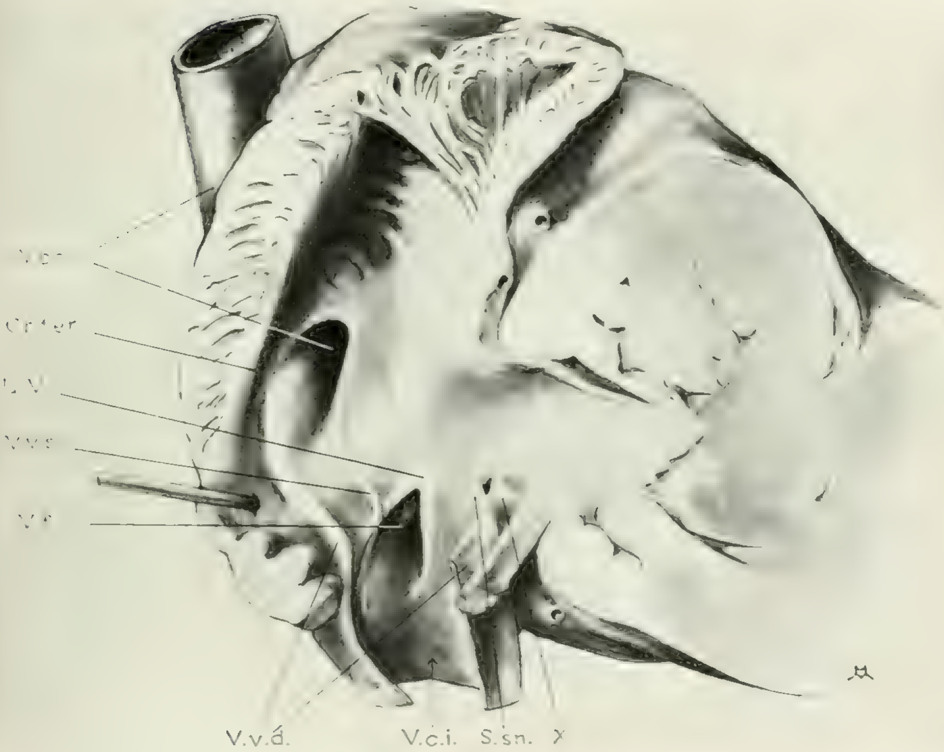
PLATE 3

EXPLANATION OF FIGURES

9 Dissection of the heart of a foetus of about 85 mm., the right atrium and right ventricle opened from the right side. The lateral wall of the inferior vena cava (*V.c.i.*) and the right sinus valve (*V.v.d.*) have been divided by a vertical cut and the parts somewhat separated. Magnified about 10 diameters.



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SOME EXPERIMENTS ON REGENERATION AFTER EXARTICULATION IN DIEMYCTYLUS VIRIDESCENS

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TEN FIGURES (THREE PLATES)

The earlier writers (Phillipeaux and Fraisse)[1] on regeneration in urodeles seem to have held the opinion that the extremities of adult animals are completely replaced only when one or more bones are injured in the amputation, that is to say, not after total extirpation (exarticulation). Wound-irritation from an injured bone was considered necessary as a stimulus to the replacement of a missing part. Also the bone supplied a 'tissue-rest' to serve as a matrix. However in young salamanders and especially in their larvae, it was found that the regeneration of extremities takes place very readily since here the joints are only partially formed and wounding of the bones always occurs in amputations. This general conclusion, that in adults regeneration does not occur after complete extirpation, seems to have been shared by a number of the more recent investigators, some, Kochs ('97) and Wendelstadt ('01 and '04) expressly confirming it, others, Towle ('01), Morgan ('03), Reed ('03) and Glaeser ('10), while not putting it to the test, seem to have taken care in their experiments to amputate through a bone.

Kurz ('12) in the course of his experiments on transplantation of entire limbs in Triton, found that if the limb is completely extirpated (exarticulated) at the hip- or shoulder-joint, a new limb regenerates. Presumably no wounding of the bones of the hip- or shoulder-girdle took place although Kurz does not state what precautions were taken to avoid this. The writer using the American salamander, Diemyctylus viridescens, obtained similar results some years previous to Kurz's report but for various reasons they were not published.[2] Recently a new series of experiments were made to work out the histological details of the process and to determine how it differs, if at all, from regeneration after injury to remaining bones or cartilages. In addition, a number of more complicated operations were made to analyze further, if possible, the extent and power of regeneration after losses not usually met with in nature.

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[1] The works of Phillipeaux and Fraisse were not accessible to the writer. Their conclusions were obtained from Barfurth's review in Merkel and Bonnet's Ergebnisse, vol. 1, 1891.

MATERIAL AND METHODS

A large supply of adult Diemyctylus was obtained through the kindly assistance of Prof. A. Treadwell, of Vassar College. Since many of these animals were in a weak, semi-starved condition when brought to the laboratory, they were kept for a month in glass aquaria before using and were fed on fresh liver. Under these conditions the animals became very vigorous, and withstood the operations well. All operations were done under narcosis. At first ether was used, but this, owing to its irritating effect on the skin and to a certain percentage of mortality which followed its use, was soon discarded. Much better results were obtained by using a solution of chloretone, of 1:2000, in which the animals were immersed. This acts very gently. After swimming around rapidly for a few minutes, the salamanders slowly come to rest and in about ten minutes are completely narcotized. The animals recover readily, though sometimes slowly after this treatment. There is no irritation of the skin and no mortality.

After the amputations, to be described in detail beyond, the best results were obtained by closing the wounds with a stitch or two of fine silk thread. Although this is not absolutely necessary to the success of the experiment, healing then takes place more rapidly and there is less danger of fungoid growths. Immediately after operation, the animals were placed in a dark chamber lined with moist filter paper for two days as recommended by Reed ('03). During this period the operated extremity was moistened from time to time with a solution of permanganate of potash 1:1000. The animals were then returned to the aquaria. The above precautions almost entirely prevented the growth of fungus and consequent failure of the experiments.

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[2] The experiment was made at the suggestion of Prof. T. H. Morgan, in 1907.

For microscopic study, the regenerating regions were removed and fixed for the most part in sublimate acetic or Gilson's mercuro-nitric fluid. Other fixatives, such as Zenker's fluid, Bouin's fluid and ten per cent formalin were occasionally used but on the whole the sublimate mixtures proved the most satisfactory. After hardening in alcohol for a few days, the objects were decalcified in a mixture of four per cent nitric acid in seventy per cent alcohol for three or four days. They were then imbedded in paraffin and sectioned. As a rule, good series were obtained, seven or eight micra thick, although the rather tough bone and cartilage from large specimens sometimes gave trouble. For staining Mayer's haemalum followed by picro-acid fuchsin was most frequently employed. This gives a brilliant differentiation of tissues but is not always permanent. Other stains such as Mallory's connective tissue stain, borax carmine and Lyons blue, haemalum and congo red were also used but none proved as satisfactory for most purposes as the haemalum and picro-acid fuchsin combination.

EXPERIMENTS

Part I

The fact that regeneration does occur after complete extirpation (exarticulation) has been established by the observations of Kurz and the writer as stated above. In order to work out the detail of this process, two sets of operations were made, the hind limbs being used in both cases. In the first set the limb was amputated at the hip-joint, in the second at the knee-joint. Great care was exercised in making these amputations. The skin and muscles were first carefully divided with a small sharp scalpel. Then the part to be removed was grasped with the forceps and slight traction employed to draw the joint surfaces apart. The capsular ligaments were then divided with the scalpel and the limb removed, care being taken not to touch the skeletal parts remaining (hip bones or femur according to the site of operation). A flap of skin and muscle was drawn over the wound and a couple of stitches taken. There was very little trouble from bleeding, but in cases where it was profuse, the specimen was discarded.

1. Amputation at the hip-joint. Nineteen animals were used for this operation divided into groups of eleven, six and two. All of the first group were killed and examined between thirty-nine and forty-six days after operation. Externally each showed a small bud at the site of amputation. Microscopically the bud was composed of a dense mass of indifferent cells with small round nuclei. No change in the hip-girdle was observed. The second group of six were kept for six months. At this time all had regenerated a new limb about three-fourths the normal size. Microscopic examination showed the normal number of skeletal elements in the limb, each represented by a bar of cartilage. There was a well developed narrow cavity in the femur and peripheral ossification had begun in all the cartilages except the tarsals which do not ossify in these animals.[3] Joint cavities were well marked at this time. The third group of two animals was lost. Owing to the small number of specimens and the lack of intermediate stages, the successive steps in this type regeneration could not be made out. The detailed account of this process will therefore be based upon the larger and more complete series of operations at the knee-joint (vid. infra).

2. Amputation at the knee-joint. About seventy-five operations of this kind were made. Most of the specimens obtained were fixed at intervals, of from ten to fifty days and sectioned. The remainder were allowed to complete their regeneration to determine whether the new part exactly resembles the old both in size and gross structure. In addition fifteen amputations were made through the distal end of the femur for comparison with the exarticulation experiments.

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[3] While it is true that peripheral ossification does not occur in the tarsalia, nevertheless an extensive marrow cavity is normally present and the irregular trabeculae of cartilage bordering it generally become calcified, if not actually bony.

Descriptive. Wendelstadt ('04) and Glaeser ('10) have given very detailed accounts of regeneration in the limbs based chiefly on species of the European salamander Triton and on the Axolotl. In Diemyctylus the process is quite similar to that observed in Triton. A study of the specimens in which amputation was made through the distal end of the femur, i.e., an operation corresponding to those of Wendelstadt and Glaeser, showed that the descriptions given by these writers for Triton, apply almost equally as well to Diemyctylus. It is true there is some slight discrepancy in their accounts but this can be discussed more conveniently when comparing the exarticulation experiments with those previously made.

The earlier changes which take place in the stump may be passed over briefly here. They are concerned chiefly with the over-growth of the integument, the breaking down of the soft parts, notably the muscle and the formation of a dense mass or bud of small cells with round, deeply staining nuclei over the distal end of the bone (femur). This mass lengthens out and forms a projection when seen externally but does not always lie in the axis of the limb. The origin of these cells could not be determined with exactness. Wendelstadt ('04) encountered the same difficulty. Towle ('01) however, states that the accummulation of 'nuclei' in the bud is due to rapid (direct) division of nuclei in the old muscle fibers and the disintegration of these fibers.[4] Undoubtedly the degeneration of muscle-fibers is largely responsible for the accumulation, but whether exclusively so or not, is difficult to decide. Connective tissue elements may also contribute something.

Turning now to the changes in the bone and cartilage with which this paper is chiefly concerned, it is here that an essential difference appears between regeneration after exarticulation and regeneration after amputation through a bone. This difference has to do principally with the behavior of the distal epiphyseal cartilage of the femur. This epiphysis (fig. 1, *Ep.f.*) becomes slowly detached from the shaft by resorption of the bone and calcified cartilage (*C.c.*) proximal to it.[5] The resorption seems to be brought about largely by the action of cells which arrange themselves along the surface of the bone or calcified cartilage and erode it. Many of them are giant cells. Their origin was not determined. Both Wendelstadt ('04) and Glaeser ('10) have described this resorption process by giant cells. Figure 1 shows the distal end of the shaft (*B.f.*) broken up into irregular fragments of bone and calcified cartilage.

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[4] Towle's experiments are concerned almost entirely with the regeneration of muscle.

Coincident with the resorption process, a change takes place in the epiphysis itself. The cartilage matrix begins to break down. This occurs first on its distal and proximal surfaces (fig. 1, *Ep.f.*). There is no evidence, however, that the cartilage cells themselves undergo degeneration. Indeed, in many instances they have been seen dividing mitotically and further, as the lacunae are opened by the degeneration of the matrix, the cells pass out and mingle with the surrounding tissues. Those liberated on the distal surface could not be further traced but on the proximal surface, that is facing the marrow cavity (figs. 1 and 2, *M.C.*), they contribute to a mass of tissue (*Ax.C.*) which is forming between the old epiphysis and the shaft. This mass which quickly takes on the appearance of young cartilage may be called axial cartilage adopting Glaeser's ('10) term.

This axial cartilage appears to have a two-fold origin: (a) from the liberated cells of the old epiphysis as stated above and (b) from the cells lining the marrow cavity and covering the trabeculae of bone and calcified cartilage at the distal end of the shaft; in other words from the endosteum. These latter cells appear to increase in number and, streaming out from the interior of the femur (fig. 1) become massed under the old epiphysis where they form the main contribution to the new axial cartilage.

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[5] It may be stated that there is normally a considerable amount of calcified cartilage at the plane of union of shaft and epiphysis. This is distinct from the uncalcified, hyaline cartilage of the epiphysis itself.

This substantiates Wendelstadt's conclusion that 'osteoblasts' (in reality chondroblasts) arising from the lining of the marrow cavity contribute to the new formation (of cartilage). Glaeser, however, derives the axial cartilage from periosteal and connective tissue fibrillae exclusively. He thinks that cartilage may occasionally arise from bone-marrow though this is doubtful. The origin of a part of this cartilage from cells of the old (distal epiphyseal) cartilage naturally could not occur in the experiments of Wendelstadt and Glaeser since the distal epiphysis was always removed in their operations. Glaeser nevertheless states that regeneration from old cartilage, either from the cells or the matrix does not occur. The writer is unable to determine from Glaeser's account upon what experiment this conclusion is based. That giant-cells in certain regions break down the matrix and open up the capsules is true but in Diemyctylus, at least, the cartilage cells certainly do not degenerate. Evidences of further activity are abundant in this material including the frequent presence of mitosis.

During the early stages in the formation of axial cartilage, the cells of the periosteum also begin to form cartilage. The site of this new formation is at first some distance from the distal end of the femur and entirely distinct from that of the axial cartilage. It may be called periosteal cartilage, or using Glaeser's term, peripheral cartilage. An early stage of its growth is shown in figure 1, *Per.C*; a later one in figure 2, *Per.C*. The formation of peripheral (periosteal) cartilage is mentioned by most workers on regeneration and there is no need to discuss it at length. In all cases it first appears at some distance proximal to the wound and gradually spreads distalward until it forms a continuous collar around the shaft reaching to the distal end (fig. 3, *Per.C.*). Cornil and Coudray ('03) described a similar formation in the healing of experimentally produced fractures in a mammal (rabbit). The first phenomena of repair, they state, are to be found at some distance from the fracture, that is the formation of peripheral (periosteal) cartilage.

Whether or not the axial cartilage always appears first could not be determined. In some cases the peripheral cartilage was distinguishable earlier while in most the two appeared at about the same time. Glaeser seems to think the axial cartilage appears before the peripheral, while Wendelstadt describes activity in the periosteum leading to the formation of peripheral cartilage before there is any outgrowth of cells from the marrow cavity to form axial cartilage. In the experiments on Diemyctylus the presence of an epiphysis may have had a modifying effect on the order of appearance. This point is probably not of any great importance.

Soon after the appearance of axial and peripheral cartilage the tissue of the bud which has formed distal to the femur, begins to show signs of differentiation. This tissue as mentioned previously is at first composed of apparently indifferent cells. Cartilaginous masses now appear in it and, since they are formed in the same manner as in normal development, they may be spoken of collectively as embryonal cartilage following Glaeser ('10). In figure 2, a mass of this cartilage (*Em.C.*) can be seen lying distal to the old epiphysis (*Ep.f.*). It is at first entirely distinct from the axial cartilage.[6] As the limb bud enlarges rapidly the growth of the embryonal cartilage keeps pace with it, the cartilage extending right into the growing tip (fig. 3, *Em.C.*) In this way the skeleton of the new leg and foot is blocked out in cartilage at a young stage, as the early experimenters found.

The formation of new skeletal parts is due chiefly to concentrations of cells in situ rather than to growth from the first formed mass or masses. There is, it is true, a continuous substratum or core of tissue running through the center of the bud from which the skeleton arises but it does not give rise to one or two continuous bars of cartilage. There are always interruptions at points where joints are to be formed.

During the growth of the embryonal cartilage, the axial cartilage continues to enlarge. This is due in part to growth at the expense of the old epiphyseal cartilage. The latter remains, however, for some time partly imbedded in the axial cartilage (fig. 3, *Ep.f*) where it can be distinguished by its different staining reaction. The expansion of the axial cartilage finally brings it into contact with the embryonal cartilage and they become so closely united as to appear almost continuous (fig. 3).

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[6] The space seen in figure 2, between the embryonal cartilage and the old epiphysis is probably an artifact.

The peripheral cartilage (fig. 3, *Per.C.*) meanwhile has spread to the distal end of the bone (*B.f.*) and there unites with the axial cartilage (*Ax.C*). This results in a continuous cap of cartilage covering the distal end of the femur, and extending proximally for some distance. Figure 4, *Ep.f.n.* from a much later stage than figure 3, shows this new cap. From it, the new epiphysis of the femur is formed and also the new bone of the intermediate zone between epiphysis and shaft which was lost in the early resorption process. A portion of the marrow cavity *M.c.n.* can be seen extending into it.[7] At *Os.n.*, new bony tissue is spreading into the cartilage. This new ossification is shown under high magnification in figure 10, which is taken from the same region of a neighboring section. The new bone (*Os.n.*) appears as a mass of interlacing fibers extending through the matrix of the cartilage (*C.n.*). In this process, the cartilage cells are gradually enclosed by the newly formed bone and converted directly into bone cells. Three such cells are shown at *Os.c.* Portions of the marrow cavity (*M.C.*) and bone of the shaft (*B.f.*) are included in the figure. Between the shaft and the marrow cavity some of the old calcified cartilage remains (compare fig. 3, *C.c.*). A direct transformation of cartilage into bone was noted by Cornil and Coudray ('03) in the healing of fractures in the rabbit. They state that in most cases the cartilage bordering the bone first ossifies along its edges while later the cartilage capsules themselves ossify, the cells being directly transformed into bone cells.

It will be recalled that a portion of the distal end of the shaft is destroyed in the resorption process by which the old epiphysis is detached from the bone. This is replaced by ossification of a part of the new cartilage in the manner just described. The further history of this process was not followed owing to lack of material in the advanced stages.

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[7] Through an error, the leader from the letters *M.c.n.* does not point to this extension.

Distally the new formed epiphysis comes into relation with the embryonal cartilage which forms the new fibula and tibia (figs. 4 and 9) and later a joint appears at this level. As previously stated, before the new epiphysis is completed and while a portion of the old epiphysis is still present (fig. 3) a close attachment is developed between the axial cartilage (*Ax.C.*) and the embryonal cartilage (*Em.C.*). It is difficult to say whether or not the two cartilages become actually continuous. The tissue uniting them is composed of cells without definite boundaries and with small elongated nuclei. It does not appear to be cartilaginous. Small cavities soon appear in this connecting tissue, beginning usually at the circumference and spreading toward the interior. An early stage in the joint formation is shown in figure 9. The new joint cavity (*J.c.n.*) is just appearing between the new femoral epiphysis (*Ep.f.n.*) and the new fibula (*Fib.n.*). In a later stage (fig. 4) the joint cavity has enlarged at the circumference and a definite capsule has been formed, the latter being continuous with the perichondrium of the new cartilages (epiphyses). A small joint cavity has also developed between the fibula and a tarsal cartilage (*T.c.n.*). At this stage the fibula itself has begun to show evidences of subdivision into shaft and two epiphysis. In the central portion, the cartilage areolae are enlarging and becoming more spherical preparatory to the formation of a marrow cavity, while at the surface a thin layer of subperiosteal bone has been laid down. In the epiphyseal portions, the cartilage cells have a tendency to arrange themselves in concentric arcs, a characteristic of epiphyses in general.

No attempt has been made in this study to arrange the specimens in a series based on time after operation. The stages of regeneration do not necessarily correspond to the intervals of time. For example figure 3 shows a stage obviously more advanced than that shown in figure 2, yet it was taken from a specimen killed thirty-eight days after operation, while the latter came from one killed at forty days. This is very probably due partly to difference in time of healing of the wound and partly to temperature. It was noticed that among animals operated on at the same time and as far as possible treated in the same manner, some always healed more readily than others. Regarding temperature—the higher degrees were in general more favorable to rapid regeneration than the lower as might be expected, but there was so much individual variation due to time of healing that no great reliance can be placed on this statement. Comparing, from the standpoint of time, regeneration after exarticulation with regeneration after wounding a bone, one finds that the former is appreciably slower. On the average, there is about ten days difference in corresponding stages.

With regard to regeneration of soft parts (muscle, nerve, blood-vessels, etcetera), there is nothing to add to the accounts already published. Muscle regeneration has been very carefully worked out by Towle ('01) in Plethodon and Schminke ('07) in Triton taeniatus and T. cristatus. These writers agree that the new muscle is formed chiefly by isolated cells (sarcoplasts) which arise from degeneration of the old muscle of the stump. The sarcoplasts are small masses of cytoplasm which contain at first several nuclei. They usually break up into small cells which are responsible according to Towle ('01) for the accumulation of nuclei (cells) in the growing bud distal to the stump of the old bone where they give rise to new muscle fibers. It was previously mentioned that the exact origin of this entire mass of small cells is somewhat uncertain (cf. Wendelstadt). Probably most of the cells originate from degenerating muscle and some perhaps from other soft tissues. In any event, there is no evidence that any are derived from bone or cartilage. Histologically there is at first no sign of differentiation in these cells, and it seems useless to assume that such exists. It is from a part of them, however, that new embryonal cartilage is developed in the midst of the bud. This seems to be an example of dedifferentiation followed by redifferentiation in the sense of Child ('15). From the apparently indifferent mass both cartilage and muscle are formed, the cartilage showing the typical embryonic type of development.

A somewhat similar process is to be seen in the behavior of the old epiphyseal cartilage of the femur. Here the matrix breaks down, liberating the cells which again become active and form new matrix. In this case, however, the cells do not dedifferentiate so far as to become indifferent; they remain cartilage forming cells. The formation of the peripheral cartilage and that part of the axial cartilage derived from endosteal cells is of course quite different in nature. Here the more or less undifferentiated cells of the periosteum and endosteum which have lain dormant, are suddenly stimulated to activity by the amputation. They form cartilage first and later a portion of the cartilage is transformed into bone as described on page 115.

It was stated at the beginning of this account that some animals were allowed to complete their regeneration to determine whether the new skeleton was like the old. There seems to be no difference whatever provided sufficient time is allowed for development. Externally, a slight deformity sometimes appears at first, since the new bud does not always lie in the longitudinal axis of the limb. This is more common after operations at the knee-joint than at the hip-joint. In the course of time this irregularity disappears and the limb becomes normal in shape and position. Complete ossification, however, may take almost a year and sometimes even longer.

A glance over the literature of regeneration in amphibia shows that the power to regenerate a new normal skeleton does not extend to all animals of this class. Morgan ('03) found that in Amphiuma the new skeleton was abnormal and deficient although some specimens were kept under observation for nearly a year. Certain results which were obtained by the earlier experimenters Goette and Fraisse seem to indicate that some of the European urodeles (Triton marmoratus and Proteus) lack the power of complete regeneration but Kammerer ('05) states that this is not the case if the animals are kept under favorable conditions and for a sufficient length of time.

In the Anura the power is much more limited. New limbs will regenerate only if amputation is made in the tadpole stage. Barfurth ('94) was the first to find that the limbs of frog-larvae (Rana fusca) are capable of regeneration, but this power disappears in the progress of development. Ridewood ('98) obtained regeneration of posterior limbs in the tadpole of the midwife-toad (Alytes obstetricans). The new skeleton was "normal or nearly so" in five cases. Byrnes ('04) using frog-tadpoles showed that the anterior limbs would regenerate while still under the operculum but the new limb is invariably smaller than normal and there is a tendency to reduction in the skeletal elements. Morgan ('08) (and Goldfarb) attempted to induce regeneration in the fore-leg of the adult frog by artificial means. Pieces of the leg, muscle and other tissues from the tail of the tadpole were grafted into the stump but with only small success. In some cases, however, incomplete regeneration of the leg with rudimentary toes was obtained, or a broad flat 'foot' with scant toes. Histological details were not given. Glaeser ('10) more recently tested the power of regeneration in the hind limbs of adult frogs but found none except in two cases where a ring of peripheral (periosteal) cartilage developed around the stump of the femur. No artificial means were used to induce regeneration in this case.

Part II

To test further the power of regeneration in Diemyctylus a series of more complicated operations were made, involving losses not usually met with under natural conditions. These are to some extent a repetition of those of Wendelstadt ('01) and Reed ('03) but with certain modifications.

Experiment 1. Extirpation of the fibula and a portion of the femoral epiphysis but without injury to the tarsus. Number of animals, ten:—Of these two escaped and one lost the foot. The remaining seven were killed at intervals of from forty-eight days to one year. There was no indication of regeneration of a new fibula, but the lost portion of the femoral epiphysis was restored.

Experiment 2. Extirpation of the fibula without injury to either femoral epiphysis or tarsus. Number of animals, ten:—These were killed at intervals of from thirty-three days to one year. For the most part no indication of regeneration was observed but in two specimens there was a narrow mass of calcified fibrous tissue or bone in the place occupied by the old fibula. This mass may have developed from fragments of the old fibular epiphysis which were accidentally left in the wound in two of the operations.[8]

The results of experiments 1 and 2 substantiate the conclusion of Wendelstadt and Reed that regeneration in a lateral direction (in the limb) does not occur.

Experiment 3. Extirpation of both leg bones and a portion of the femoral epiphysis but without injury to the tarsus. Number of animals, five: –Considerable shortening occurred but in no case did the foot drop off. One was killed after sixty-three days and the remainder after one year. Two of the latter showed some indication of regeneration. In these there were one or two cartilaginous nodules, in one case fairly extensive, connected with the femoral epiphysis by fibrous tissue. The femoral epiphysis itself was restored in all.

Experiment 4. Extirpation of both leg bones but without injury to either femoral epiphysis or tarsus. Number of animals, seven:—Shortening of course occurred. Two lost the foot subsequently and were discarded. Of the five remaining, one was killed at sixty days and the remainder after one year. All showed definite attempts at regeneration, in some cases quite well marked. Figures 5 and 6 from the same specimen cut in the dorsi-ventral plane show the extent of regeneration in the best marked case. The time elapsed was sixty days. Only a small portion of the femoral epiphysis (*Ep.f.*) and one tarsal cartilage (*T.c.*) appear in figure 5, since the section lies near the border of the limb. The new skeletal element of the leg (*L.s.n.*) consists of an epiphyseal portion which articulates with the femur and a long, narrow bar of cartilage which is partly overlaid by bone at either end. (The bone is darkly shaded in the figure.) At the proximal end a distinct joint capsule with cavity (*J.c.n.*) has developed. Figure 6, from a section near the median plane, shows another portion of the new skeletal element (*L.s.n.*). This portion is a solid mass of cartilage united to the femoral epiphysis (*Ep.f.*) by a capsule with joint cavity (*J.c.n.*) as before. Distally it is in contact with a tarsal cartilage (*T.c.*) but no joint capsule has developed. The two portions of the new element are continuous proximally when traced through the series. Unfortunately intermediate stages in the formation of the new skeletal element were not obtained. The epiphysis of the femur (fig. 6, *Ep.f.*) has the appearance of new cartilage similar to that of the new element (*L.s.n.*). This seems to indicate that the regeneration was centrifugal in direction and probably occurred in the same manner as described in the first section of this paper. No changes were observed in the tarsal cartilages.

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[8] In the experiments described in part II, all bones removed were examined under a binocular microscope to determine whether any part of them had been accidently left in the wound.

Figure 7 is from another specimen killed after one year and cut in a plane passing through the borders of the limb. The new elements here consist of two large masses of cartilage (*L.s.n.*) united by fibrous tissue and connected with femoral epiphysis (*Ep.f.*) by a capsule containing a joint cavity (*J.c.n.*). Distally the new cartilages fall short of the tarsus. The tarsal cartilages themselves (*T.c.*) show signs of growth in a proximal direction (centripetal regeneration). They have become united proximally by a mass of cartilage which, however, has no connection with the new skeletal elements. The arrangement produces what may be called a soft joint.

Other specimens in this experiment showed the formation of irregular masses or nodules of cartilage but not so extensively as the two described above. There appears, then, to be a limit to the power of regeneration under the conditions of the experiment. This may be due to an inhibiting influence from the presence of the foot and to shortening of the limb which leaves very little room for the new growth. It may be well to state that as soon as the wound heals, the animal uses the limb constantly when creeping over the bottom of the aquarium. Wendelstadt ('01) performed a similar experiment upon the anterior limb of the Axolotl but with entirely negative results though he kept the animals under observation for ten to fifteen months. The limbs shortened as in the case of Diemyctylus but the animals apparently made no attempt to use them. It is just possible that a certain amount of activity in the limb is necessary to start the regenerative process. Wendelstadt also tried the effect of leaving a small piece of one of the bones (ulna) in situ. For this operation he used one Axolotl and one Triton. In the latter the humerus was also wounded. The axolotl regenerated a new ulna which was shorter than normal while in the Triton a whole new forearm and a second hand were formed. This peculiar malformation in the Triton was never duplicated in any of the writer's experiments on Diemyctylus, although in some cases the femur was purposely wounded. It is improbable that there is any difference in the power of regeneration of fore and hind-limbs in these animals.

Experiment 5. Extirpation of the fibula and removal of the foot entire without injury to the femoral epiphysis or tibia. Number of animals, five: In this lot two were killed at sixty-six and ninety-five days respectively and the remainder at the end of a year. The first of these had regenerated a well-marked foot when killed and a new fibula. The latter consisted of a solid bar of cartilage with a layer of subperiosteal bone surrounding its proximal two-thirds. It was attached to the femoral epiphysis by a capsule, common to it and the tibia. Distally it was connected with the new tarsalia by ligaments, in places showing the beginning of a joint cavity. In the specimen killed at ninety-five days, the foot had regenerated but there was scarcely any indication of a new fibula. The remaining three specimens killed after one year regenerated a new complete foot including tarsalia and a new but incomplete fibula. A section through one of these is shown in figure 8. The old tibia (*Tib.*) articulates with the femoral epiphysis (*Ep.f.*) while the new fibula (*Fib.n.*) falls short proximally. Peripheral ossification has started in the new element but there is no marrow cavity as yet. The new tarsalia are seen at *T.c.n.* The distal epiphysis of the tibia seems to be composed of new cartilage like that of the tarsals and fibula. This is to be expected since it was shown that the old epiphysis in a stump is always replaced by new cartilage (vid. Part I). In the present experiment one would expect first a new formation of cartilage from which a new tibial epiphysis and the skeleton of the new foot is formed. This is followed by growth in a proximal direction to form the new fibula (centripetal regeneration). Apparently the energy of regeneration is not always sufficient to produce a complete fibula even in a year's time although it may do so in two months as in the first specimen described (sixty-six days).

A tendency to regenerate centripetally was also noted in experiment four, where both leg bones were removed and the foot allowed to remain. In this case however it was limited to the formation of a mass of cartilage (fig. 7, *T.c.*) uniting the proximal surfaces of the tarsalia. Wendelstadt ('01) obtained centripetal regeneration in the axolotl by extirpating the upper ends of the radius and ulna. In three animals, the bones were completely restored after fifteen to eighteen months. There was apparently no tendency to regenerate centrifugally from the femoral epiphysis.

Morgan ('08) using Plethodon and Diemyctylus tried to discover what kind of a structure would regenerate from the proximal end of a limb. For this purpose the limb was cut off and grafted onto the stump in reverse position. Regeneration occurred but results were complicated, due to mixing of old and new material and to the turning of the graft in the skin-pocket. This method was discarded in favor of the following: The hind leg was cut off at the knee. Then the femur was cut off high up in the thigh and the distal portion reversed in position. A new limb regenerated. Its skeleton was composed of (1) proximal stump of femur, (2) connecting cartilage, (3) piece of reversed femur, (4) new tibia and fibula, and (5) foot. There was considerable evidence of absorption and in only a few cases did it seem probable that the material for the new limb came from the exposed proximal end of the grafted piece. In some cases the new cartilage from the proximal stump grew past the graft. On the whole it is not quite clear from Morgan's account what part the graft played in regeneration, as histological details are not given.

Reed ('03) performed a series of experiments somewhat similar to those just described (Exp. 5) except that the distal end of the tibia was removed with the foot. Spelerpes ruber was used for these experiments. The results were, regeneration of the distal end of the tibia, a new foot and a new fibular element. The latter was usually incomplete but in one case it almost completed itself proximally, that is, reaching the femoral epiphysis. As in the present experiments there was no tendency to regenerate from the femur. Wendelstadt, in his later paper ('04) states that the experiments of Reed confirm his general conclusion that wounding of the skeletal elements is necessary for regeneration. The experiments described in the present paper show that this conclusion is too sweeping. It is true that if one bone only (fibula or radius) is removed (Wendelstadt, Reed and the writer) or if the proximal parts of two bones are removed (Wendelstadt) no regeneration occurs from the uninjured epiphysis of the femur (or humerus). In the first case, the pressure of the remaining bone against the joint surface of the femur (or humerus) and the tarsals (or carpals), that is the presence of a functional joint may inhibit regeneration from these points. In the second case the new growth centripetally from the remaining injured bones, which is always more rapid than from uninjured ones, may make up the deficiency in time to check any tendency to regenerate from the epiphysis of the humerus. Shortening of the limb which must occur in this case would also be a factor. These, of course, are mainly suggestions. Further experiments are necessary before definite explanations can be made.

SUMMARY AND CONCLUSIONS

1. In Diemyctylus regeneration takes place readily after complete extirpation (exarticulation) whether the operation is made at the hip- or knee-joint (Part I), or at the ankle-joint (Part II, Exp. 5). The time elapsed is somewhat longer than when a skeletal element is injured.

2. The new skeletal elements are similar to the old. There is no tendency to reduction.

3. The essential difference between regeneration after exarticulation and regeneration after wounding a skeletal element lies in the behavior of the cartilaginous epiphysis which is present

in the stump in the former case. This cartilage becomes detached from the shaft, gradually breaks down and is, partly at least, reconverted into cartilage which assists in the formation of a new epiphysis.

4. The new cartilage which forms the basis for the skeletal elements appears independently in three localities:

a) Around the shaft of the bone proximal to the epiphysis (peripheral cartilage). This cartilage is periosteal in origin.

b) In the axis of the bone and in contact with the marrow subsequent to detachment of the epiphysis (axial cartilage). The origin in this case is twofold: (1) From the cells of the old epiphyseal cartilage and (2) from the lining of the marrow cavity (endosteum).

c) In the tissue of the bud distal to the epiphysis (embryonal cartilage). Here dedifferentiation appears to have taken place forming a substratum of indifferent cells from which in turn new cartilage is formed as in early development of the limb.

5. If a single bone (fibula) is removed completely from the leg, it is not replaced either by proliferation from its fellow (lateral regeneration) or from the skeletal elements lying proximal and distal to it even when one of the latter is injured.

6. When both leg bones are completely removed they are replaced to some extent by new elements which, however, are always irregular and incomplete. The origin of the new parts was not definitely determined.

7. When one leg bone (fibula) and the foot are removed without injuring any of the remaining skeletal elements, a new complete foot is regenerated from the distal end of the remaining leg bone (tibia). This is followed by a slow and often incomplete regeneration of the lost leg bone (fibula) in a proximal direction (centripetal regeneration).

LITERATURE CITED

Barfurth, D. 1894 Sind die Extremitäten der Frösche regenerationsfähig? Arch. f. Entw.-Mech., Bd. 1.

Byrnes, E. 1904 Regeneration of the anterior limbs in the tadpoles of frogs. Arch. f. Entw.-Mech., Bd. 18.

1904 On the skeleton of regenerated anterior limbs in the frog. Biol. Bull., vol. 7.

Child, C. M. 1915 Senescence and rejuvenescence. Univ. of Chicago Press.

Cornil, V. et Coudray, P. 1903 (Note) De la formation du cal. Comptes rendus de l'Academie des Sciences, Tome 137, p. 220.

Glaeser, Karl 1910 Untersuchungen über die Herkunft des Knorpels an regenerierenden Amphibienextremitäten. Arch. f. mikr. Anat., Bd. 75.

Kammerer, P. 1906 Die angeblichen Ausnahmen von Regenerationsfahigkeit bei den Amphibien. Zentralbl. f. Phys., Bd. 19.

Kochs, W. 1897 Versuche uber die Regeneration von Organen bei Amphibien. Arch. f. mikr. Anat., Bd. 49.

Kurz, Oskar 1912 Die beinbildenden Potenzen entwickelter Tritonen. Arch. f. Entw.-Mech., Bd. 34.

Morgan, T. H. 1903 Regeneration of the leg of Amphiuma means. Biol. Bull., vol. 1.

1908 Experiments in grafting. Amer. Nat., vol. 42, No. 493.

Reed, M. 1903 Regeneration of a whole foot from the cut end of a leg containing only the tibia. Arch. f. Entw.-Mech., Bd. 17.

Ridewood, W. G. 1898 On the skeleton of regenerated limbs of the midwife-toad (Alytes obstetricans). Proc. of Zoöl. Soc. of London.

Schmincke, A. 1907 Die Regeneration der quergestreiften Muskelfasern bei den Wirbeltieren. Verhandl. d. phys.-med. Gesell. zu Würzburg. Bd. 39.

Towle, E. 1901 On muscle regeneration in the limbs of Plethodon. Biol. Bull., vol. 2.

Wendelstadt, H. 1901 Über Knochenregeneration. Arch. f. mikr. Anat., Bd. 57.

1904 Experimentelle Studie über Regenerationsvorgänge am Knochen und Knorpel. Ibid., Bd. 63.

PLATES

PLATE 1

EXPLANATION OF FIGURES

Figures 1 to 4 are from specimens in which complete amputation of the limb was made at the knee-joint.

1 Longitudinal section of a limb 30 days after operation. *Ep.f.*, femoral epiphysis; *Ax.C.*, axial cartilage; *Per.C.*, peripheral cartilage; *C.c.*, calcified cartilage of the shaft; *B.f.*, bone of the shaft (femur); *M.c.*, marrow cavity. The narrow space distal to the epiphysis is an artifact. Magnified about 30 diameters.

2 Longitudinal section of a limb 40 days after operation. *Em.C.*, embryonal cartilage. Other abbreviations as in figure 1. The space between the femoral epiphysis, *Ep.f.*, and the embryonal cartilage *Em.C.*, is probably an artifact. Magnified about 30 diameters.

3 Longitudinal section of a limb 38 days after operation. Abbreviations as in figures 1 and 2. Magnified about 30 diameters.

4 Longitudinal section of a limb 48 days after operation. *T.c.n.*, new tarsal cartilage; *Fib.n.*, new fibula; *J.c.n.*, new joint-cavity; *Ep.f.n.*, new femoral epiphysis; *Os.n.*, bone formation in new cartilage; *M.c.n.*, extension of the marrow cavity into the new cap of cartilage (see foot-note on p. 115); *B.f.*, bone of the femur. Magnified about 30 diameters.

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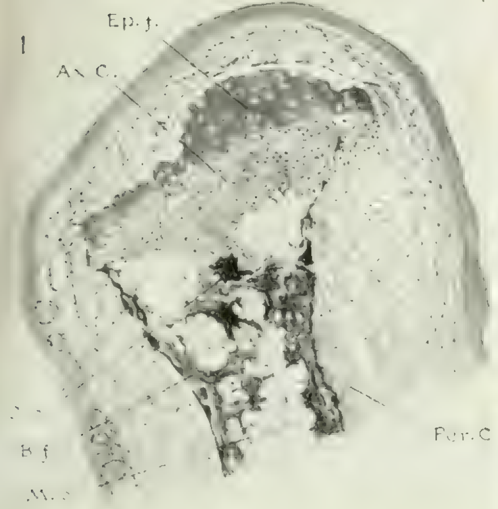

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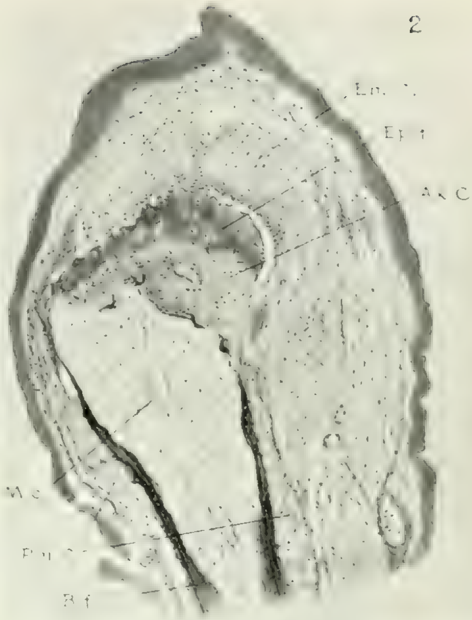

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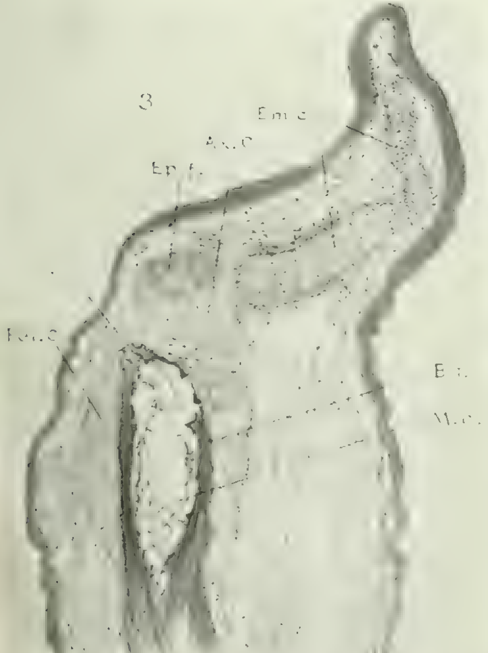

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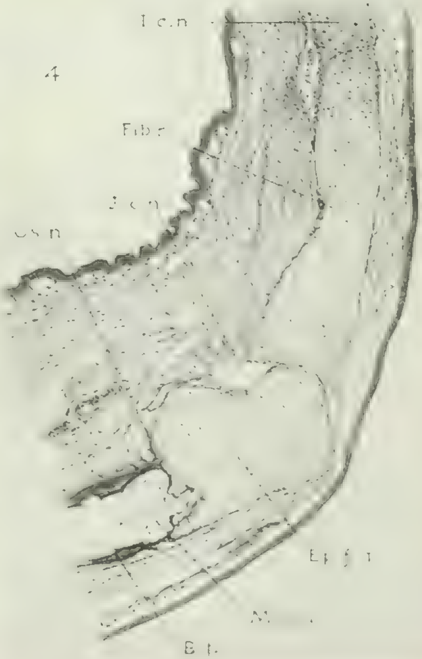

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PLATE 2

EXPLANATION OF FIGURES

5 and 6 Longitudinal sections of a limb from which both tibia and fibula were completely removed. Time after operation, 60 days. *T.c.*, tarsal cartilage showing normal marrow cavity and partly calcified lining; *L.s.n.*, new skeletal element of the leg, in figure 5, partly ossified; *Ep.f.*, epiphysis of the femur; *J.c.n.*, new joint cavity; *M.c.*, marrow cavity and *B.f.*, bone of the femur. Magnified about 30 diameters.

7 Longitudinal section of another specimen after same operation as above. Time one year. Tarsal cartilages blended proximally, *T.c.;* new skeletal elements *L.s.n.* Other abbreviations as in figures 5 and 6. Magnified about 30 diameters.

8 Longitudinal section of a limb from which the foot and the fibula were completely removed. Time after operation, one year. *T.c.n.*, new tarsal cartilages; *Fib.n.*, new fibula (incomplete); *Tib.*, tibia; *Ep.f.*, femoral epiphysis. Magnified about 30 diameters.

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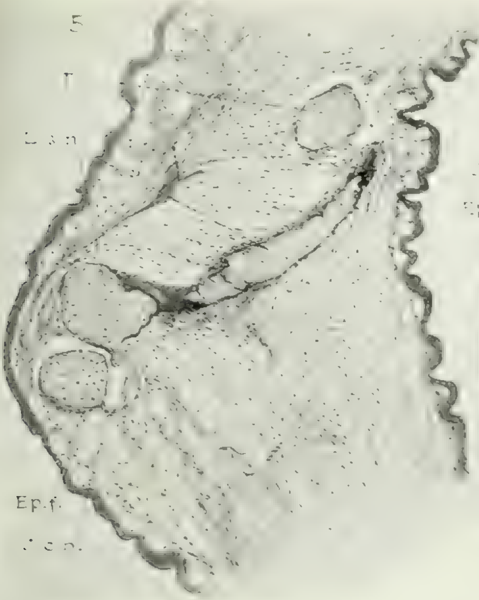

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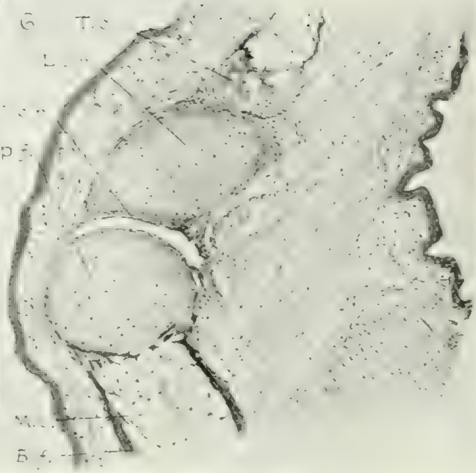

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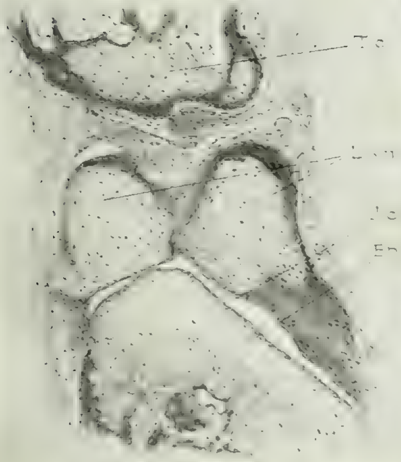

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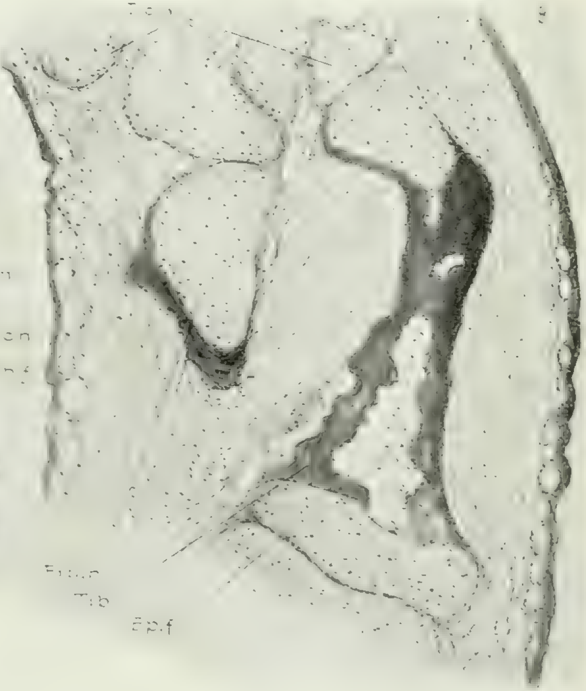

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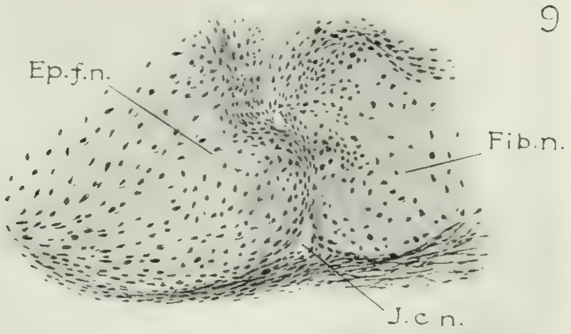
PLATE 3

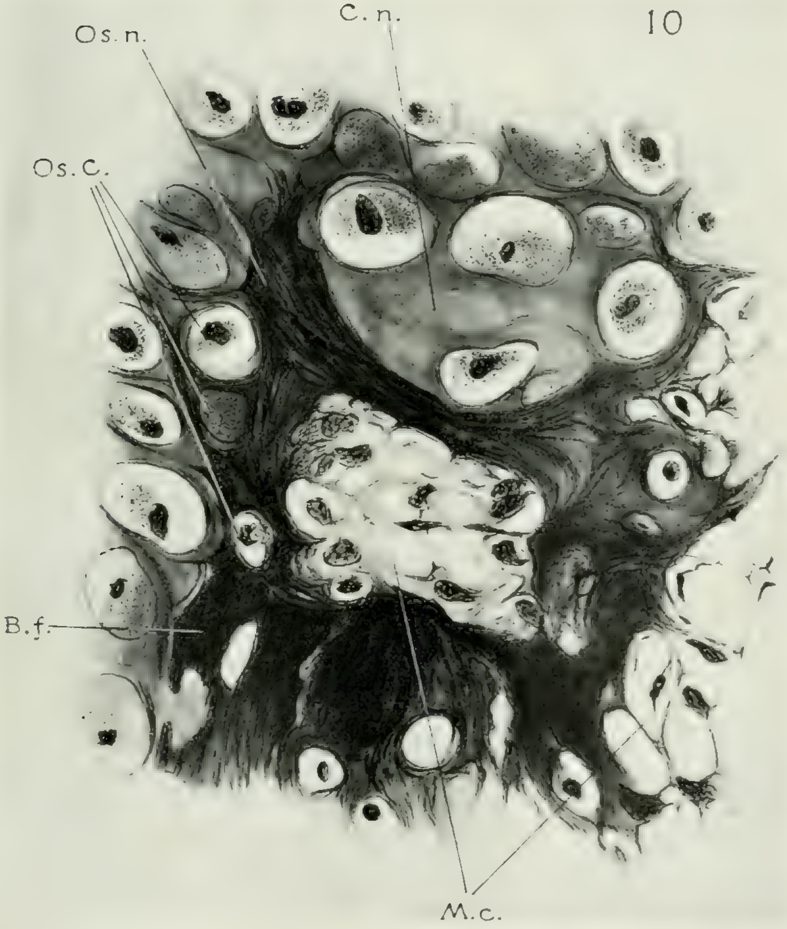
EXPLANATION OF FIGURES

Figures 9 and 10 are from specimens in which complete amputation of the limb was made at the knee-joint (exarticulation).

9 From a longitudinal section of a limb 41 days after operation. *Ep.f.n.*, new femoral epiphysis; *Fib.n.*, new fibula, proximal end; *J.c.n.*, new joint-cavity forming. Magnified about 100 diameters.

10 From a longitudinal section of a limb 48 days after operation. *C.n.*, new cartilage; *Os.n.*, new bone spreading through the cartilage; *Os.c.*, new cartilage cells transforming into bone-cells; *B.f.*, old bone of the shaft (femur); *M.c.*, portions of the marrow-cavity. Magnified about 420 diameters.





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MICRODISSECTION STUDIES. I.

The Visible Structure of Cell Protoplasm and Death Changes

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A study of the physical properties of protoplasm requires for its foundation a knowledge of the consistency of protoplasmic structures in the living cell. Barber's pipette holder, a mechanical device for manipulating microscopically fine pipettes and glass needles in a hanging drop has proved most valuable for this purpose as with it one may dissect living cells under direct microscopic observation. The technique used will be described in a paper which will shortly appear. One must be fully alive to the difficulty of discriminating between protoplasmic structures and artifacts frequently produced during dissection. Considerable experience was, therefore, found to be necessary for a proper interpretation of the results obtained.

Marine invertebrate and plant ova lend themselves well to microvivisection as their post-operative development is easily followed. The bulk of this paper, therefore, deals with results obtained from studies on the protoplasm of Asterias and Arbacia ova at Woods Hole and that of Echinarachnius, Cerebratulus and Fucus ova at South Harpswell.

It is remarkable how much tearing and pulling with a needle living protoplasm will undergo without showing injury. One may puncture a cell with a needle and drag the needle through the cytoplasm back

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[1] This paper is based on the work of two summers at Woods Hole Marine Biological Laboratory, and one summer at the South Harpswell Laboratory. The writer wishes to express his indebtedness to Prof. F. R. Lillie for accommodation at Woods Hole and to Prof. H. V. Neal for facilities accorded at South Harpswell. An abstract was read before the American Physiological Society, December, 1916.

and forth cutting through the sides of the cell and if the procedure be slow and gradual the tear closes up behind as the needle proceeds and the process may be continued almost ad libitum without producing an ill effect. If, on the other hand, the needle be carried rapidly through the cytoplasm, a few thrusts only are necessary to induce rapid disorganization. The effects of injury are probably cumulative. If injurious effects be made to follow one another without giving the cell time for recovery, the additive effects of the injury soon manifest themselves in disorganization of the protoplasm resulting in the death of the cell.

The cytoplasm. In the marine eggs studied, the protoplasm consists of a hyaline fluid matrix in which are imbedded granules of various sizes. The fluid offers no perceptible resistance to the needle and an indication of its very slight viscosity lies in the fact that when the needle is moved through the fluid, the only granules displaced are those in the immediate vicinity of the needle. The fluid is water miscible. If, for example, the cell surface be torn, the interior cytoplasm pours out and mixes completely with the surrounding water. Also, if a drop of water be injected slowly and gradually into the egg by means of the mercury injection method, the water diffuses throughout the cytoplasm diluting it. A dilution can be produced in this way sufficient to produce Brownian movement of the imbedded granules. The surface film of such an egg is so much weakened that a mere touch suffices to burst it open when everything except the smallest granules disappears in solution. This water soluble hyaline protoplasm coagulates with ease on mechanical injury. Mere compression will often cause an egg to coagulate into a solid mass. It can then be cut into pieces which hold their shape. This is apt to lead one to the erroneous conclusion that the substance of a cell is usually a solid protoplasmic gel.

When a concentrated aqueous solution of neutral red or brilliant cresyl blue[2] is injected into the interior of an egg, the dye spreads from the tip of the pipette diffusely staining the hyaline cytoplasm. Within a few seconds a granule here and a granule there begins to stain. In a short time all the dye is taken up by the scattered granules leaving the cytoplasm colorless. At the tip of the needle where the concentration of the stain is at its greatest all the granules finally stain. Elsewhere colorless granules lie scattered among the colored ones. The stain is

[2] For the use of the brilliant cresyl blue and for noting the effect of acetic acid on the macrosomes, I am indebted to Mrs. M. R. Lewis.

never permanent. Within a few minutes to an hour, depending on the amount injected, the stain completely fades away. A continuously stained condition can be maintained only by the presence of a superfluity of the dye.

The granules in the cytoplasm of the eggs studied vary considerably within certain limits in size (fig. 1). They have been described by E. B. Wilson (2) and Kite (6). One may classify them into two groups. The smallest granules or microsomes, as they may be called, are minute specks considerably below 1 μ in diameter but plainly visible with the high powers of the microscope, their refractive indices differing considerably from that of the surrounding cytoplasm. The larger granules or macrosomes (Wilson's alveolar spheres), vary from 2 to 4 μ in diameter and may be circular, oval or irregularly polygonal in shape, exhibiting simple outlines with no diffraction rings. They are closely crowded throughout the egg and among them are scattered the microsomes. The macrosomes, constantly but in almost imperceptible degrees, change in shape and position and it is probable that they are as constantly disappearing and reappearing in the hyaline cytoplasmic matrix. They are very translucent in Echinarachnius and so contribute to the remarkable transparency of the egg. In the Cerebratulus egg they are comparatively opaque.

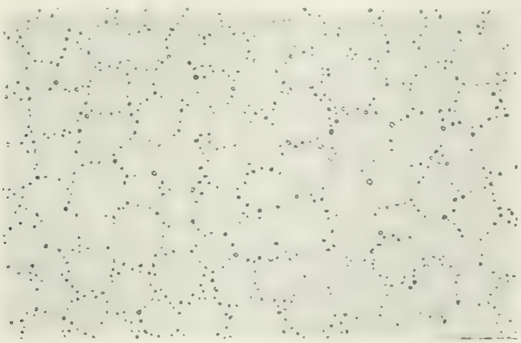

Fig. 1. Illustrating the protoplasm of a living Echinarachnius egg with microsomes and translucent macrosomes (the latter 2 to 4 μ in diameter) crowded together in a hyaline fluid.

The difference between the micro and macrosomes is brought out most prominently on injury of the cell by acids or by the needle. If a hanging drop of sea water containing eggs be subjected to the fumes of acetic acid for a few seconds, the macrosomes quickly disappear leaving the microsomes in a hyaline coagulated mass of cytoplasm. The dissolution of the macrosomes occurs also when disorganization of the protoplasm is induced by rapidly repeated tearing of the cytoplasm with the dissecting needle. The macrosomes swell and gradually but rapidly fade into the surrounding cytoplasm which now becomes very liquid and in which the microsomes exhibit a dancing Brownian movement. The disorganized area then spreads on all sides, and, if the initial injury be extensive and no protective membrane intervenes (see farther on), soon involves the entire cell. If the injurious tear be such as to destroy the surface film in one spot, the disorganized liquefied cytoplasm flows out and mixes completely with the sea water and, in a few seconds the entire cell, except for the dancing microsomes, disappears from view. The microsomes appear to be the most resistant structures of the cell.

Wilson's conclusions (2) that there exists "a complete gradation in size from the largest alveoli" (macrosomes, as I call them) "down to the microsomes" is based, I believe, on a misconception derived from crushing protoplasm. On crushing protoplasm, the macrosomes go into solution and the disintegrating protoplasm as it flows out, tends to break up into spherules of varying sizes, by the formation of surface films. These spherules vary extremely in size and may fuse with one another on touching. They swell readily in water or may coagulate and are quite different from the macrosomes of the uninjured egg. Kite (3) describes the formation of more or less rounded masses in dying Asterias eggs and rightly distinguishes them from structures normally present in protoplasm.

Frequently before all the disorganized cytoplasm has poured out and mixed with the surrounding water, the outflow ceases and the whole mass changes into a solid coagulum which may or may not show a network structure. The complexity in constitution of the protoplasm must account for the fact that a very slight difference in the manner of injury, or in the state of the protoplasm, at the time of injury may produce such widely differing end results, viz., a coagulation into a solid jelly or a complete dissolution into the surrounding medium. The coagulum consists of a hyaline gel with granules arranged in a delicate meshwork. Occasionally upon disintegration the cytoplasm begins to set before the macrosomes have quite gone into solution. Such a mass often resumes dissolution with a succession of peculiar spasmodic jerks apparently due to the macrosomes which, here and there, swell and carry into solution regions surrounding them.

In cells killed by fixing reagents, the death changes take place without destruction of the cell wall. If the fixing agent act rapidly, the macrosomes tend to swell simultaneously and coagulation sets in before the microsomes are irregularly dispersed. The precipitation network incident to coagulation is produced regularly and the result is a fairly symmetrical alveolar structure with microsomes imbedded in the alveolar walls. Flowing may occur in the disintegrated cytoplasm if the fixing agent be slow in producing coagulation and a variety of distorted figures may result. The prolonged action of acetic or hydrochloric acid tends to dissolve the microsomes and, with various fixing reagents, a variation in coagulation effects occurs so that one obtains granular and network precipitates which may simulate protoplasmic structure but in reality completely mask it and often lead to erroneous conclusions regarding cell inclusions.

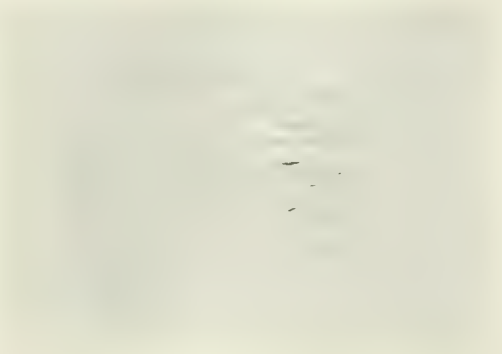

Fig. 2. Drawing of a sector of an Echinarachnius egg in the astral stage fixed in Bouin's picroformol and stained with iron hematoxylin. The effect of fixation is seen in the fibrous appearance of the astral rays and in the network structure of the coagulated cytoplasm.

The cell aster is a structure whose nature is entirely masked on fixation. In the living cell the rays are probably paths due to a centripetal flow of a hyaline fluid in an otherwise temporarily gelatinized cytoplasm. When a cell containing an aster is fixed, the reagent used disorganizes the cytoplasm in the way described above. The rays are then compressed between artificially produced alveoli, and their substance being precipitated, gives the appearance, familiar in fixed material, of fibers or rows of granules which seem to merge peripherally into an alveolar network (Fig. 2).

An acid reaction of protoplasm on mechanical injury can be demonstrated in the Arbacia egg where many of the macrosomes are deep reddish brown chromatophores. On injury to the cell, the color diffuses into the liquid cytoplasm, changing from brown to an orange pink hue,[3] denoting a distinct increase in acid reaction. Also in cells whose granules are stained wth neutral red, injury causes the dye to diffuse out of the macrosomes as they dissolve and give to the liquid cytoplasm a rose color. Acid fuchsin also, which is avidly taken up by acid regions, stains the injured disorganized cytoplasm while the normal cytoplasm remains colorless.

The peripheral layer of the cell. The surface layer of the egg cells studied is very dense in consistency as compared with the cell interior into which it merges insensibly. In the unfertilized egg, the cell granules are imbedded in it up to the very line of division between the egg and surrounding medium. With the needle the surface may be pulled out into long strands without otherwise disturbing the contour of the cell. On being released the strands tend to curl and retract slowly till they disappear. If a more rapid tear be made, and if the cell be under compression, the spot torn bulges out as the internal cytoplasm presses on the weakened surface. The surface layer of the swelling protuberance is very easily broken, upon which the interior may pour out. The cytoplasm then either disintegrates entirely in the surrounding water or, if remaining normal, reëstablishes a film on its surface. When left undisturbed the new surface film gradually strengthens into a definite ectoplasmic layer and the protuberance slowly retracts until the original contour of the egg is reëstablished. If the point of attachment of the protuberance be small, the protuberance may be pinched off to form a spherule of cytoplasm which to all appearances is normal.

If a tear of the surface layer or of any part of the cytoplasm be so injurious that disorganization sets in, a film may form around the disorganized area separating it from the sound cytoplasm. The recovery of an injured cell is only brought about by the formation of a membrane-like film which prevents extension of the injury. A succession of films may form, as one after the other, they succumb to the steady advance of the destroying process and the film which finally holds out may enclose only a fraction of the original cell but what it encloses will be normal protoplasm. The retention of the forming surface film is aided by the presence of solid structures in the cytoplasm. These act as bases which shorten the span to be bridged over by the new film. In the mature fertilized egg, the developing sperm aster which is a gelatinized ball of cytoplasm frequently acts in this way.

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[3] This color reaction can be demonstrated in an aqueous suspension of the echinochrome made by drawing off a quantity of the colored body fluid into distilled water.

When the surface layer is injuriously torn and the disorganized interior cytoplasm flows out, the torn edges of the surface layer curl out and rapidly dissolve, upon which the entire cell disappears in the surrounding water. If the disorganization of the interior has been produced without destruction of the surface layer, the surface often becomes transformed into a rigid coagulum enclosing the fluid products of the disintegrated protoplasm.

The readiness with which the surface film can be reformed is seen in the following experiment on the unfertilized mature Arbacia egg. If an egg be compressed in a hanging drop and then pushed along with a blunt needle, peculiar currents can be produced in the egg substance. The currents pass directly from the pushing object in a straight line through the egg to the anterior end where they curve outward and flow back along the surface to be caught again in the flow from the pushing object. The egg surface is thus being continually reformed by an outflow of its interior, much as the falling sides of a fountain of water are formed by the jet that is streaming up at the centre. The readiness, however, with which the internal cytoplasm may be transformed into the substance of the surface layer is limited. If the egg be pushed beyond this limit no surface layer forms and the cytoplasm at once disorganizes and dissolves in the surrounding water. If one stops before reaching this limit, the egg can be made to round up and will continue a normal existence.

In protozoa, the surface layer of protoplasm, the ectoplasm, is very pronounced. If the ectoplasm of Paramoecium bursaria be torn, internal pressure causes the endoplasm to flow out through the tear which is at first a gaping hole in the ectoplasm. The contractility of the ectoplasm, however, is such that the torn rim curves in. In this way the free edges of the tear tend to approach and if the tear be slight they meet and further outflow of the endoplasm is stopped. In time the concavity on the surface produced by such an injury fills out and the cell resumes its normal shape. If the gap in the tear be too wide for this method of repair, a surface film bridging the gap forms which may break repeatedly until the outflow lessens the internal pressure sufficiently to allow the newly formed film to persist. Within a few hours, evidence of the tear is no longer visible, either the film stiffens into a definite ectoplasmic layer or the entire body of the cell contracts to bring the original edges of the gap together making the ectoplasm again a continuous layer. A paramoecium may be readily cut into pieces by squeezing it between the coverslip and a fine glass rod, or a knife which is not sharp enough to cut through the ectoplasm. The knife edge bears down upon the surface of the cell until the floor of the groove formed touches the ectoplasm on the other side. The surfaces of contact fuse and further pressure of the knife separates the cell into two portions possessing no gap through which the fluid endoplasm may escape. The cautions usually given in the technique of cutting up protozoa is significant when we bear the above in mind. The knife must be as sharp as possible (which will still be blunt from the point of view of the paramoecium), the cutting edges must be free from nicks and, in cutting, the experimenter must bear down upon the knife without giving it a drawing movement. Either of the two latter precautions, if unheeded, prevents a continuous surface of contact for the ectoplasmic layer of opposite sides and when the knife is removed the endoplasm will flow out through the gap thus destroying the cell. In the marine ova studied, the ectoplasmic layer is very thin but the same condition holds true, viz., that cut pieces will persist only when the cut surface can be bridged over by a morphologically definite film.

In summary, we may say that the surface layer is a highly extensile, contractile and viscous gel capable of constant repair. Its establishment and maintenance is a property essential to protoplasm. With the film intact the mass of protoplasm maintains itself and the life of the cell is assured. When the film is destroyed the cytoplasm flows out, the macrosomes swell and disappear, the whole mass completely disorganizes and disappears in solution in the surrounding water.[4]

In regard to the difference in permeability of the surface layer of a cell and its interior, I have so far only tested the diffusion of the three basic dyes, neutral red, cresyl blue and janus green (6). A droplet was injected into the cell simultaneously with the application of a similar droplet to its external surface. For all three dyes diffusion into the cytoplasm took place equally rapidly whether applied to the egg surface or injected into the interior.

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[4] Kite describes the cytoplasm of the Asterias eggs as "a quiet translucent gel which can be cut into small pieces with comparative ease." His paper is a pioneer one in microdissection research. The observations recorded were necessarily fragmentary and the differences between the surface layer of the egg and its interior as also the ease with which protoplasm forms gel membranes escaped his notice.

Insect germ cells. The germ cells of certain insects, Periplaneta, Disosteira and Anasa, were studied both in modified Ringer's fluid and in the serous fluid of the insects used (3, 4). By pricking the walls of the cysts in the testes, spermatocytes in different stages of development flow out as isolated cells. In the serous fluid they may be kept alive two or three days, during which all stages of cell division may be observed. Except for the nucleus and the mitochondrial network which surrounds it, the resting cell consists of a hyaline fluid cytoplasm. By very slow action with the needle, strands of protoplasm may be pulled out which retract on being released. Injury, however, very easily manifests itself upon which the cell outline fades and the cytoplasm disappears in solution in the surrounding medium.

Adult somatic cells. These vary greatly in consistency owing probably to the amount of metaplasmic material into which their protoplasm has been transformed. Of the various types, only a few possess the fluid consistency of embryonic and germ cell protoplasm. With the exception of the leucocytes, they are comparatively resistant to mechanical injury and are tough and fairly rigid bodies. The nerve cell is probably a rigid gel. The muscle fiber is also a gel. Its substance can be easily pulled out into strands which retract completely when released. Continued injury causes the muscle substance to pass into a very rigid hyaline gel which may be cut into discrete non-glutinous pieces. Gland cells swell readily when punctured and torn with the needle. They seem to exist both in sol and gel states. Mucosa cells consist of a soft, very extensile gel. They tend to round up when isolated. The leucocyte possesses a protoplasm which is very like the undifferentiated protoplasm of the germ cells. If a leucocyte with pseudopodia be touched with the needle, the pseudopodia are retracted and the cell becomes spherical. If the tip of a very fine needle be inserted gradually into the leucocyte a puncture may be made without apparent injury. The diminutive size of the cell, however, renders necessary very little accummulation of injurious effects to disorganize the cell. This usually occurs with almost explosive rapidity, the entire cell going into solution. Dead leucocytes are coagulated bodies and may be cut into non-glutinous pieces.

The cell nucleus. Observations on the cell nucleus of all the ova studied agree with those of Kite (5) that the resting nucleus is hyaline and exists in the sol state. In the germinal vesicle of the immature egg a definite membrane seems to bound the nuclear substance. It is extensile but easily destroyed. Evidence that this membrane is a morphological structure is shown on withdrawing some of the nuclear contents with a micropipette. The nucleus then partially collapses throwing the nuclear surface into irregular folds. Suspended in the nuclear fluid in which it may be pushed about with ease is the germinal spot or nucleolus distinctly visible on account of the difference in its refractive index from that of the nuclear fluid. It frequently contains one or more vacuoles and does not appear to be solid for it may be cut into two, each part rounding up like a droplet.

A rapid tearing of the germinal vesicle with the needle point produces an injury which is accompanied by some remarkable changes. The nucleolus at once swells and fades from view, the nuclear membrane entirely dissolves and, as the nuclear fluid comes into contact with the surrounding cytoplasm, immediate disintegration takes place. The destructive action spreads and may involve the entire egg unless a protective film forms to enclose the area of destruction as in a vacuole. A vacuole of this kind gradually works to the surface of the egg, where it is eventually extruded and expelled. When this has occurred the egg resumes its normal appearance although smaller than before and minus its nucleus. The disintegrative action of the nuclear substance very quickly disappears on extraction from the cell. It is, however, possible, by acting rapidly, to produce the destruction of one cell by injecting into it the substance of the germinal vesicle of another cell. If the nuclear substance remains more than five or ten seconds in the micropipette it is found to be innocuous on injection. With the normal breakdown of the germinal vesicle in the maturing egg, the cytoplasm acquires an increased sensitiveness to injury by the needle. This sensitiveness gradually passes off during the polar body formation.

In the mature egg both nucleus and cytoplasm are comparatively resistant to injury. The nucleus is a hyaline sphere which behaves like an immiscible fluid drop in the cytoplasm. One may divide it into two and each part rounds up into a droplet. On coming into contact the droplets run together. Such a process is no hindrance to normal nuclear activity, for in one case an egg so treated was subsequently fertilized and segmented normally. Rapid thrusts of the needle into the nucleus cause injury, which manifests itself either in a swelling followed by complete dissolution or a rapid coagulation with the production of a granular meshwork precipitate simulating the nuclear network of fixed cells.

SUMMARY

1. Protoplasm is a hydrophilic colloid which, in early germ cells, egg cells and Protozoa, usually exists in the sol state with a surface layer in the gel state. Adult somatic cells generally are gels in which one cannot demonstrate a cell membrane possessing a consistency different from that of the cytoplasm within.

2. The microscopically visible granules in the cytoplasm of the egg of Arbacia may be classified into two groups: (a) The microsomes, which are considerably less than one micron in diameter and constitute the most resistant parts of the cell maintaining themselves after complete disorganization of the cell; (b) the macrosomes, which range from 2–4 micra in diameter and are very sensitive to injury.

3. The external surface of the egg cell is a gel which passes gradually into the sol in the interior. The surface gel is very extensile and contractile and is readily regenerated on injury. Tearing of this surface, if unrepaired, results in the pouring out of the internal cytoplasm and dissolution.

4. A remarkable property of protoplasm is its ability to form a protective gel film not only on its external surface but also around an injured area which is in the process of disorganization. The disorganized mass thus insulated is eventually expelled from the cell.

5. A continuous but gradual application of mechanical injury can be sustained by a cell for some time without evidence of harm done. A short but rapid application produces instant local destruction, the spread of which may involve the entire cell.

6. Disorganization of the cytoplasm of the egg cells studied takes place in the following way: First, the macrosomes swell and go into solution, and second, the liquid hyaline cytoplasm may flow out and disappear in the surrounding water or it may suddenly set forming a rigid coagulated mass. The coagulation structure gradually coarsens with the production of a network or granular precipitate.

7. Injury is accompanied by a swelling and an apparent increase in the acid reaction of the part involved.

8. The comparatively rigid ectoplasm and the fluid endoplasm of Protozoa are directly comparable with the surface layer and the internal cytoplasm respectively of the marine ova studied.

9. The surface layer and the internal cytoplasm appear to be equally permeable to the basic vital dyes used.

10. The germinal vesicle of an immature egg consists of a hyaline liquid enclosed in a gel like membrane. The nucleolus is an immiscible droplet floating in the vesicle and is very sensitive to mechanical injury.

11. The contents of the germinal vesicle of an immature egg, if brought into contact with egg cytoplasm, either by mechanical rupture of the vesicle or by injection, produce instant destruction of the cytoplasm. This is not true for the mature nucleus nor for the segmentation nucleus.

12. The cytoplasm of an immature egg is comparatively impervious to mechanical tearing, that of a mature egg is very much more sensitive.

13. In the mature egg the nucleus behaves as a fluid droplet whose substance is immiscible with the cytoplasm. It may be divided into two droplets which unite on touching. It coagulates with ease on mechanical injury.

BIBLIOGRAPHY

(1) Chambers: Biol. Bull., 1917, xxxii.
(2) Wilson: Journ. Morph., 1899, Suppl. to xv, 1.
(3) Chambers: Science, N. S., 1914, xl, 824.
(4) Chambers: Science, N. S., 1915, xli, 290.
(5) Kite: This Journal, 1913, xxxii, 146.
(6) Kite: Biol. Bull., 1913, xxv, 1.

ADDENDUM

If the injection of an aqueous solution into an egg be not very carefully and gradually done (see page 2) the mechanical compression caused by the force of the injection will produce a coagulation film about the injected droplet to form a vacuole. This film exhibits diosmotic properties similar to that of the gelled surface of the egg for, as Kite observed (6), such a vacuole filled with hypertonic sea water increases in size while one containing distilled water loses its water and decreases in size.

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MICRODISSECTION STUDIES

II. THE CELL ASTER: A REVERSIBLE GELATION PHENOMENON[1]

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ONE PLATE

I. INTRODUCTION

1. Historical

The periodic appearance and disappearance of the aster in the cell, the very definite structure which it offers to the eye, and its very evident relationship to cell division make it one of the most striking phenomena in cell protoplasm.

Any idea which one may advance regarding its structure is necessarily based on a conception of the structure of protoplasm. There can be, therefore, as many interpretations of the astral structure as there are theories of the physical make-up of protoplasm. Taking for a basis the reticular theory of protoplasm, the astral rays have been considered fibrous strands whose radiate arrangement is produced by being drawn together at a point in the protoplasmic fibrous network. In accordance with Bütschli's foam theory of protoplasm the aster has been explained as an arrangement of the protoplasmic alveoli into more or less definite rows radiating from a common center.

The experimental work of Morgan ('08, '10), Lillie ('09), Conklin ('12), and others on the centrifuged eggs has shown conclusively that whatever be the structure of protoplasm the mitotic spindle at least is of such a consistency that it preserves its structure on being driven through the cell substance The astral radiations often persist about the poles of the spindle being apparently dragged along with them (Spooner '11). F. R. Lillie ('09), suggests that the aster about the poles of a dislocated spindle is a new configuration, and due to forces (see Hartog '05), which are focussed in the centrosomes and influence the cytoplasmic granules in situ. A significant phenomenon is the occasional occurrence of distortion of the astral rays not only in centrifuged but also in normal eggs (Mark '81, Coe '99, Conklin '02, and Painter '16). Investigators generally agree in considering the distortion to be a proof for the existence of more or less extensive movement in cell protoplasm and some conclude that the rays may be firmer in consistency than the surrounding cytoplasm.

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[1] This paper was read before the American Society of Zoologists, New York City, December 27, 1916. It is based on the work done during the summer of 1916. The writer wishes to express his indebtedness to Prof. H. V. Neal, for accommodation and facilities accorded him at the South Harpswell Laboratory.

In a recent paper Heilbrunn ('15) describes in the cytoplasm of sea urchin eggs a gelatinization produced by chemical agents which cause the eggs to undergo parthenogenetic development.

While investigating the structures of various marine ova by micro-dissection, the author found it possible to detect changes in the consistency of the protoplasm during aster formation and to demonstrate that during cell division we meet with definite reversible gelation phenomena.

2. *Material and method*

The microdissection method, first introduced for the study of protoplasmic structures by Kite (Kite and Chambers, '12), affords means of collecting direct evidence of the physical consistency of many constituents in the living cell. The apparatus used for holding the dissection needle is the mechanical pipette-holder of Barber. A detailed description of the instrument and of ways for making the micro pipettes and needles is given by Barber in his paper ('14). An elaboration of the method for microdissection purposes is being published (Chambers, '17). A preliminary description has been given (Chambers, '15).

The needle is drawn out of hard glass tubing. It is manipulated with Barber's instrument and projects into a moist chamber on the stage of the microscope. The tip of the needle is bent at right angles so that it can be brought into a drop hanging from the roof of the chamber. In the drop are the cells to be dissected.

Marine eggs were selected whose development could be easily followed so as to determine their post operative viability. The eggs of Echinarachnius and of Cerebratulus lend themselves well for operative work, that of Echinarachnius because of its beautiful transparency and that of Cerebratulus because of its comparatively high resistance to injury. In Echinarachnius the presence of red chromatophores, sparsely scattered throughout the jelly surrounding the egg, is not enough to disturb one's view and the jelly and fertilization membrane are too soft and extensile, during very early segmentation periods, to afford any hindrance to the microdissection needle. The Cerebratulus egg is rather opaque and the egg membrane which rises off the surface of the egg when placed in sea water is comparatively tough. The membrane, however, can be easily removed by passing the eggs, which have stood in water for twenty minutes to one-half an hour, through a fine cambric cloth. It was also found that the eggs could be considerably flattened during cell division, so that the various cell structures are spread out in a broad, comparatively shallow area and the asters form with rays very long in the plane of flattening and very short in other directions. This offers a distinct advantage for dissection purposes.

3. *Hyaloplasm and hyaloplasm-sphere*

The word hyaloplasm is used in this paper to designate the continuous hyaline ground substance of protoplasm in which granules (the microsomes and macrosomes or alveolar spheres of Wilson) are embedded This use of the word hyaloplasm, as applied to the interalveolar material of Bütschli's protoplasmic foam, is urged by Wilson ('01 a, b.) because of its priority and also because it is purely descriptive and introduces no theoretic implications.

The word hyaloplasm-sphere (Wilson, '01) is given to the central transparent area of the aster from which the astral rays radiate. In fixed material Wilson describes this area as including the central clear zone (heller Hof of Boveri) surrounding the centrosome and the adjacent zone which contains no alveolar spheres but in which radii are closely crowded together. In the living object no trace of radiation appears in any zone of the hyaloplasm-sphere and Wilson himself raised the question whether the rays seen in its outer zone in sections may not be coagulation products. Neither is any structure resembling a central body or centrosome visible.

Eggs which have been kept flattened for some time in a shallow hanging drop tend to undergo pathological changes. The hyaloplasm-sphere is quickly affected by the abnormal conditions so that frequently cytoplasmic granules (usually the microsomes only) enter the sphere and arrange themselves in more or less regular and concentric rings. Sharply defined radiating lines appear only in coagulated eggs. In some the lines start from the very center of the sphere, in others they not reach the center leaving a central clear area which do develops a finely granular or aveolar structure. Thus several of the types of centrosomal structures figured by Wilson in his book on the cell ('00, p. 310) can be simulated in the Echinarachnius eggs by subjecting them to abnormal conditions.

In the living egg the microdissection method discloses a decided difference in consistency between the substance of the hyaloplasm-sphere and the hyaloplasm surrounding the sphere. I shall substitute in this paper the term 'sphere' for the hyaloplasm-sphere and refer to the contents of the sphere as the sphere substance or sphere liquid.

4. Protoplasm

When protoplasm is examined with transmitted light it appears as a hyaline perfectly homogeneous substance in which granules may or may not be embedded. In ova the granules are fairly uniform in size and usually so crowded together as to give to the cytoplasm of the egg the appearance of an alveolar structure. That the visible granules are not a constant feature of protoplasm may be seen in early germ cells (Chambers, '15) where the protoplasm is optically homogeneous. Very young ova also are free of visible granules which appear only as the eggs grow when they accumulate until the egg substance is crowded with them (Wilson, '99). That the hyaloplasm is really heterogeneous in structure, although under ordinary microscopic examination it appears homogeneous, can be demonstrated if, instead of using transmitted light, the hyaloplasm be illuminated by a beam of light striking it from the side only. The presence of minute particles large enough to intercept and scatter the light rays are then revealed through the production of a cone-shaped beam of light known as the Faraday or Tyndall phenomenon.[2] If the particles are not resolvable (in which case they are known as amicrons) a hazy light is all that can be seen. If, however, the particles are large enough (when they are known as submicrons) they appear to the observer as shining spots dispersed throughout a transparent medium. In the liquid hyaloplasm, these particles exhibit active Brownian movement (Gaidukov, '10, Marinesco, '12, Price, '14). In coagulated hyaloplasm they are motionless.

Because of its ultra-microscopic heterogeneity in structure, we speak of protoplasm or hyaloplasm as existing in the colloid state to distinguish it from 'crystalloid' or true solutions, whose ingredients are too small to disperse light rays of visible wave lengths, and from true solids in which no Brownian movement is discernible. In a colloid solution the dispersed particles constitute the internal or dispersed phase and the liquid in which they are suspended is the external phase or dispersion-medium.

Of the two great classes of colloidal solutions, the class consisting of emulsion colloids[3] is of chief interest in biological phenomena. To this class belong all the colloids obtainable from organic matter, such as gelatin, glue, albumen and starch. They are colloids in which both phases may be liquid, the internal phase being probably more viscous than the external. They are not readily coagulated with salts, their two phases can be separated only with considerable difficulty and they possess the peculiar property of existing in liquid or in solid or pectinized states, either one of which is capable of being transformed into the other. The liquid state is called a sol and the solid state a gel. The ability of passing one into the other is spoken of as a reversible action. In the sol the internal phase is in a highly dispersed condition. In the gel the degree of dispersity of the internal phase is decreased to a minimum until in all probability the dispersed phase passes into a continuous phase (Hatschek, '16, p. 63), the two phases of the colloid becoming, so to speak, locked one in the meshes of the other.[4]

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[2] The Faraday phenomenon is the principle upon which the ultramicroscope is based.

[3] The other class is that of the suspension colloids in which the dispersed phase consists of solid particles suspended in a liquid dispersion-medium. Suspension colloids have no viscosity and do not, as a rule, undergo gelation. Their two phases are comparatively easy to separate and they are readily coagulated with salts. Examples of this class are the metallic suspension-colloids or suspensoids.

In protoplasm we meet with properties which are peculiar to solutions of colloids of the emulsoid class,[5] having water as the basis of its dispersion-medium. It is more viscous than a true solution, water diffuses through it with readiness (Chambers, '17), and it is capable of forming reversible sols and gels (see this paper, experimental parts).

Bütschli based his theory of the alveolar structure of protoplasm on a comparison of protoplasm with experimentally produced foam structures. He observed the striking similarity between the microscopic picture of a finely divided emulsion of xylol in a soap solution and a protoplasmic network. He also pointed out that variations in the viscosity of protoplasm could be explained by such an emulsion structure.[6] Optically homogeneous protoplasm he interpreted as possessing alveoli the walls of which are either so thin as to be invisible or possess a refractive index similar to that of the surrounding substance ('02, p. 217).

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[4] This process may continue until a complete reversal of the phases takes place, the originally internal phase becoming the external phase and vice versa. The internal phase of an emulsion-colloid or emulsoid being more viscous than the external phase (which is aqueous), a reversal of the phases produces a solidifying of the diphasic system.

[5] It is significant that the vast majority of organic emulsion-colloids are hydrophilic.

[6] An emulsion of oil droplets in a soap solution is liquid when the oil exists in a comparatively low degree of dispersity. On sufficient shaking the dispersity of the oil is raised and the surface of contact between the emulsified oil and the emulsion's medium is so enormously increased that the resulting surface tension solidifies the mass.

Regarding the gross structure of protoplasm, Bütschli's interpretation of an alveolar structure is true only for special cases where the microscopic appearance of a meshwork is due to the inclusion in the hyaloplasm of droplets or granules which are not fundamental to protoplasm (Mathews, '07, Kite, '13, Chambers, '17). In killed protoplasm the visible meshwork is distinctly a coagulation phenomenon and has nothing to do with the structure of living protoplasm (Chambers, '17). Bütschli's interpretation applies rather to protoplasm in connection with its ultra-microscopic, colloidal structure and even then probably only to its gelled or coagulated condition.

The protoplasm of certain marine ova (Asterias, Arbacia, Echinarachnius, Cerebratulus) has been described in a recent paper (Chambers, '17 a). It consists of a hyaloplasm in which clear more or less spherical bodies of 2 to 4 micra in diameter, the macrosomes, and very small granules, the microsomes, are crowded together. The hyaloplasm of the resting egg is in the sol state and is of such a slight viscosity that the nucleus and cell granules can be readily rolled and pushed about in it by the needle. At the egg surface the hyaloplasm is in the gel state and there seems to be no doubt that protoplasm owes its high viscosity, extensibility and contractility to the gelatinized condition of its surface film.

In experiments which involve tearing of protoplasm one must recognize a limit to the amount and character of the mechanical disturbance which the general protoplasm can bear without disorganization. Considerable displacement without injury to the protoplasm can be produced by a slow even movement of the needle. If, however, the movement be quick and rapidly repeated, disorganization with a change to a decidedly acid reaction occurs accompanied either by a liquefaction or an irreversible coagulation of the disorganized protoplasm.

II. EXPERIMENTAL

1. The Aster

The fully formed aster (cf. one of the asters in the illustration of the amphiaster in figure 10) in the Echinarachnius egg[7] consists of a central, hyaline, more or less spherical area, the sphere, from which radiate an enormous number of narrow rays consisting apparently of the same hyaline substance as that of the sphere. The rays are broadest at their base and gradually taper along their course to lose their identity before quite reaching the margin of the cell. The cell nucleus lies in the sphere somewhat to one side of the center (fig. 7). The granular cytoplasm surrounds the hyaline rays and projects between them into the sphere in the form of shorter or longer conical processes (fig. 1). The sphere consists of a clear non-viscous liquid. The needle may be moved about in it without a disturbance of the surroundings. Before the nucleus has increased much in size, it may be pushed about with ease in this liquid area. On the other hand, the cone-like projections of cytoplasm are very solid in comparison. They may be bent (fig. 2) and pulled about and act as comparatively rigid gelatinous structures with the cytoplasmic granules immovably embedded in them. The rays consist of a liquid similar in appearance and consistency to that of the sphere. The cytoplasm through which the rays extend is solid when contrasted with the liquid cytoplasm of resting eggs. In short, dissection of a cell containing a fully developed aster gives one the impression that the cytoplasm is in the gel state, while the sphere and its rays are liquid.

The gel state is most pronounced in the cytoplasm bordering the sphere, its rigidity diminishing as one approaches the periphery of the cell. The comparative stability of this gel state allows of considerable distortion without a destruction of the aster. At the height of the astral stage the aster may be twisted into a spiral or other distorted shapes. On removing the needle it may or may not resume its original shape. Figure 3 is a diagrammatic representation of a distortion produced by inserting the needle into the aster of a flattened egg and moving the needle for a short distance in a straight line. The pectinized strand-like masses of cytoplasm lying around the liquid rays are pulled at an angle by the needle, producing a like distortion in the channels which are occupied by the rays. Figure 4 shows how the gelatinized strands drag after the needle when the needle is inserted to one side of the nucleus and the nucleus is pushed along for some distance. A local destruction of a few rays may be produced by rapid thrusts of the needle, a reversal of the gel into the sol state taking place. On removal of the needle the injured area appears in sharp contrast to the surrounding area. Gradually, however, it gels and acquires its original radiate appearance.

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[7] The aster in Cerebratulus, Arbacia and Asterias agrees essentially with the above description.

More extensive tearing of the aster causes a complete disappearance of its ray-like structure the entire cell reversing from the gel to the sol state. If this has not injured the cell protoplasm the sphere will retain its identity as a clear area in the granular cytoplasm. This can be made to disappear by churning the cytoplasm with the needle. If the needle be then removed, or if it be held stationary, the fluid collects into a sphere, hyaline rays again appear with their bases merging into the sphere, the gel state of the surrounding cytoplasm reappears and the fully reformed aster occasionally continues its normal course of development. Usually, however, destruction of the aster is followed by an abnormal reformation, the rays being very irregular or absent in some regions. Cell division is then much impaired or completely inhibited.

Attempts were made to break up the sphere into several parts with a view of producing several asters. The centrosphere may be broken up into several areas but when left undisturbed and if the cytoplasm remains normal these areas generally fuse together to form a single aster. However, eggs which had remained in a very compressed condition in a slowly evaporating hanging drop often spontaneously produce asters. Abnormal conditions, under which class may be included the treatment of eggs for the production of artificial 'astrospheres' (Morgan, '96, '99) and cytasters (Wilson, '01 a and others including Yatsu, '05) possibly cause a localization of the sphere liquid in several centers instead of allowing a flow into a single sphere.

2. The formation of the aster

The development of the aster was studied from the moment of its appearance on the entrance of the sperm into the egg, until the egg had segmented into eight cells and numerous dissections were made in the various stages. Because of the beautiful transparency of the Echinarachnius egg the different stages of the aster were best followed in this form and the following remarks, unless otherwise stated, apply to it.

Within two or three minutes after the entrance of the sperm and in the vicinity where it entered, a tiny aster appears which rapidly grows in size and extent. From the beginning it contains a liquid center of appreciable size. That the cytoplasm about this center has been rendered fairly solid is shown by the fact that the aster at this stage may be pushed and rolled in the surrounding liquid cytoplasm (fig. 5). The sperm nucleus is held in the gel around the sphere (fig. 6), and is therefore dragged about with the aster. The aster grows while at the same time the sphere increases in size evidently by a centripetal flow of liquid in the rays. The cytoplasm lying between the rays increases in rigidity as the gel state gradually extends in area.

During this time the eccentrically placed aster moves to the egg center. The shifting in position is possibly due to unequal pressure exerted at its periphery as the aster extends in area. An equilibrium obtains when the aster finally comes to rest at the center of the egg. As the sphere increases in size the sperm nucleus comes to lie in it. The nucleus may be dragged out of the sphere but as long as it is within the confines of the aster, it will, on being released, move slowly back into the sphere. As the aster slowly moves towards the center of the egg, its growth extension is heralded by a streaked appearance of the surrounding cytoplasm. The streaks run in the direction of the converging rays and are due to a gradual gelation of the granular cytoplasm between the paths of an exuded liquid flowing into the sphere. The coming together of the egg- and sperm-nuclei was observed to occur in the following manner: As long as the egg-nucleus is beyond the confines of the aster, that is, while it still lies in the liquid cytoplasm, it is stationary. When the extending rays of the aster reach the egg-nucleus streaks of gelatinizing cytoplasm appear all about it. The nucleus then moves toward the sphere with increasing rate until, at the border of the sphere, it works into the liquid area where it comes to lie closely pressed against the sperm-nucleus. The movement of the egg-nucleus may be explained by assuming that there exists a centripetal current of the fluid in the rays. That such a current exists may be inferred also from the following experiment: In an egg one may occasionally see one or more small oil-like droplets 2 to 4 micra in diameter. They are possibly due to degenerative processes in the protoplasm. If one of these oil-like droplets be pushed by the needle from the liquid cytoplasm into the periphery of the aster it will move along the rays toward the center.

3. *The amphiaster*

The large fusion nucleus occupies an excentric position in the hyaline central area of the aster (fig. 7). The appearance of being partially enveloped by the sphere liquid as by a cap is enhanced by the gradual increase in size of the nucleus. Very soon the ray-like structure of the cytoplasm begins to fade from view (fig. 8). The disappearance of the rays commences in what will be the equator of the dividing egg and gradually extends to the two poles. That the disappearance of the rays is due to a reversal of the gel to the sol state of the cytoplasm is shown by the fact that the needle on being dragged through the cytoplasm, now carries no distorted strands in its wake but pushes the granules about as if they were embedded in a liquid which was only slightly viscous. In the cases observed a few of the rays extending from the tips of the two poles in the plane of the future longitudinal axis of the cell never faded entirely from view. During this stage, which lasts a few seconds, the nucleus acquires a squatty bipyramidal shape with its broad equator spreading quite across the sphere. The sphere then takes on the shape of two semilunar areas lying over the two poles of the nucleus (fig. 9). The rays soon blaze out again but this time around two centers, the two polar areas, producing a brilliant amphiaster (fig. 10). The rays extend almost to the periphery of the egg and with the needle it is possible to show that the cytoplasm is again a comparatively rigid gel.

As the astral formation extends into the equator of the amphiaster the gelled strands of the two asters become irregular and distorted in shape where strands from opposite directions meet (fig. 10). The gelation figure here is a meshwork of strands rather than linear strands such as form between the rays elsewhere. In the immediate vicinity of the nucleus, however, the strands form continuous lines and since many lie directly on the nucleus, give it a fibrous appearance. No fibers of any kind could be distinguished within the substance of the nuclear spindle.

Soon after the amphiaster has fully formed the bipyramidal nucleus lengthens into a long oval body (fig. 10). Its bulging equator gradually retracts until its sides are fairly parallel. At this stage in Toxopneustes (Wilson and Mathews, '95) Wilson describes a pause of some duration during which the astral radiations become very much reduced in extent. In Echinarachnius the asters and the cytoplasm remain in the gel state although the rays appear to be less brilliant than hitherto. After a space of time (about ten minutes) a change takes place in the cytoplasm about the equator of the nuclear body which appears to be a reversal to the sol state. I was not able to convince myself on this point by the use of the needle. The cytoplasmic granules, however, which were hitherto held in a gel, become visibly mobile in the equatorial region where the rays of the two asters meet. As a constriction in the equator of the nuclear body deepens and widens a distinct flow of the granules medianward can be observed, that is, into the region of the constriction (fig. 11). Following this a constriction in the equator of the cell takes place and one gains the impression that this is consequent to a liquefaction of the cytoplasm in the equatorial plane while elsewhere it remains in the gel state. The liquefaction is accompanied by a loss in the ray-like structure in this region. During the remainder of cell division, a one-sided appearance obtains for each daughter aster, the rays being most prominent at the polar end and absent in the region corresponding to the equator of the mother cell (fig. 13). By the time cell-division is completed all of the cytoplasm has returned to the gel state, each cell containing a single aster, the nucleus lying to one side within the sphere. This brings us back to the same figure as in the undivided egg with a single aster (fig. 7). In the segmentation of the blastomeres the process as described is repeated.

The amphiaster may be twisted in much the same way as the single aster. Figure 12 represents a distortion of the amphiaster produced by dragging the needle across the equator of the cell and pulling the highly viscous nuclear 'spindle' along with it. On removing the needle, the asters may assume their original appearance. Where a distortion persists abnormalities in cell division occur.

4. The cell nucleus

Various suggestions have appeared in print regarding the relation between the formation of the fibers of the nuclear spindle and of the aster. Mention may therefore be made here of some of the results obtained in the microdissection of the nucleus during cell-division. A paper dealing with nuclear structure will be published shortly.

The spherical nucleus, which lies between the poles of the amphiaster, possesses a plainly visible nuclear membrane. This has already been described by Wilson ('95) in Arbacia. As the nuclear body becomes bipyramidal (in the metaphase) and elongates (in the ana- and telophases) the boundary between it and the surrounding cytoplasm fades from view. It preserves its identity, however, as a structure which is optically homogeneous except for the chromosomes which lie in its substance. Manipulation with the needle gives one the impression that it is a comparatively rigid body. It is gelatinous and somewhat extensible. Figure 12 shows how it may be pulled about on the cytoplasm dragging the poles of the amphiaster with it. The extensibility of its substance is shown in figure 16. Figure 15 represents a maturation figure in the Cerebratulus egg. The astral figure at the end of the 'spindle' directed toward the surface of the egg adheres to the cell periphery. The other end lies free in the liquid cytoplasm. In figure 16, the spindle is shown pulled out and bent by a needle which has been inserted into one end On being released the spindle may slowly return to its original shape.

Spindle fibers have not been detected as morphological structure until death coagulation sets in. May they not be interpreted as strands produced by the coagulation of a hyaline substance in which, previous to coagulation, there existed lines of stress or diffusion?

In the telophase the nuclear mass becomes dumb-bell shaped owing to a constriction around its middle. The constriction deepens and broadens as the two halves of the nuclear body draw away from one another and a strand connecting them does not always break through until the cell itself has completed its division.

5. *The aster in maturation figures*

This was studied in the Cerebratulus egg. The germinal vesicle breaks down twenty minutes to half an hour after the egg has been placed in sea water. Its place is taken by a clear area in the granular cytoplasm which flows gradually in one direction to the periphery of the egg spreading somewhat as it goes. In this columnar or rather funnel shaped area appears the first maturation spindle (fig. 14). As the spindle elongates hyaline plasma collects on its two poles around which the astral rays become more and more distinct while the cytoplasm between the rays passes into the gel state (fig. 15). The whole structure, viz., asters and spindle, can be pulled and pushed about causing the rays to form curves and spirals. If the distortion be not too great, the spindle will continue its normal development irrespective of spirally curved rays which may or may not straighten out. The ray-like structure of the aster is difficult to distinguish in the hyaline cytoplasm at the peripheral pole as the refractive index of the hyaline protoplasm even in its gel state is close to that of the rays. The rays of the peripheral aster, however, very soon reach the periphery of the egg. As the cell periphery is probably a protoplasmic gel it and the peripheral aster form a continuous gel, firmly anchoring the spindle. With the needle one may stretch the spindle (fig. 16) but it cannot be dislodged without causing disorganization.

Division of the nuclear spindle is followed by a reversal of the astral structure to the sol state. The sol state of the cytoplasm lying immediately under the surface where the polar bodies are to form is so liquid that granules pushed into it exhibit active Brownian movement. On the surface at the middle of this liquid region the egg bulges forming a nipple-like protuberance. This occurs in such a way as to give one the impression that the protuberance is due to a local weakening in the consistency of the surface where an internal pressure causes cytoplasm to flow out. As one of the daughter nuclei of the first maturation figure lies directly under this spot, it is carried into the protuberance which pinches off to form the first polar body. By pricking with the needle one can so affect this surface as to cause it to produce in succession five and six or even ten protuberances, each being pinched off in its turn and very closely simulating polar bodies. The other daughter nucleus of the first maturation figure now lies free in the protoplasm (fig. 17) and can be pushed about anywhere in the egg. If it be moved out of the hyaline area into the granular cytoplasm and left there (fig. 18), the granules immediately surrounding it gradually move away until the nucleus comes to lie again in a hyaline area (fig. 19). Granules, in the meantime, invade the original hyaline area until it is indistinguishable from the rest of the granular cytoplasm. The nucleus now elongates and migrates again towards the periphery of the egg, astral rays reappear about its poles and, when the second maturation spindle is fully formed, one finds it again firmly attached to the surface of the egg. The second polar body is thus produced some distance away from the first one (fig. 20).

III. DISCUSSION

The pioneer observers of mitotic division, for example, Auerbach, O. Hertwig, Bütschli and Fol, described the accumulation of hyaline plasma at the astral centers and suggested that the astral radiations are a result of protoplasmic currents. Later investigators, such as, Rhumbler ('96, '99), Morgan ('00), Wilson ('01) and Conklin ('02), were convinced that centripetal currents do occur in the formation of the aster. Experimental evidence described in this paper confirms these views. It is necessary, however, to make a distinction between the general hyaloplasm and the substance constituting the astral rays and sphere. The formation of the aster consists in the gelation of the hyaloplasm which comes under the influence of the astral center. A hyaline liquid separates out during the gelation and flows in innumerable centripetal paths toward the center where it accumulates to form the sphere. This centripetal flow brings about an arrangement of the gelled hyaloplasm containing the cell-granules into radial strands separated by the hyaline liquid paths. This produces the astral figure. The strands of gelatinized cytoplasm merge peripherally into the surrounding liquid cytoplasm or reach and anchor themselves in the substance of the gelled egg surface when the aster is fully formed. The liquid rays merge centrally into the substance of the sphere, the liquid of the rays and of the sphere being thus identical.

The term ray could be applied to the gelled strands as well as to the liquid paths. However, the liquid paths are tributaries of the central sphere of the aster and it appears very likely that they condition the radiate appearance of the gelled cytoplasm. It seems appropriate, therefore, to limit the use of the specific term ray to the centripetal paths as I have done in this paper.

At a meeting of the American Society of Zoologists in December, 1916, before which this paper was read, Heilbrunn ('17)[8] presented results of investigations on cell division which make his conclusions and mine confirm one another rather strikingly. By the centrifuge method Heilbrunn found that after fertilization the cytoplasmic viscosity rises gradually until it reaches a maximum. It is at this time that the mitotic spindle first makes its appearance. The appearance of the spindle is followed by a gradual decrease in viscosity and the egg cytoplasm returns to its original liquid state. With the microdissection method I was able to locate the gelatinized region in the forming aster. It is significant that Heilbrunn's conclusion, arrived at by using the centrifuge and by noting the effects of reagents which increase or inhibit cell gelatinization, and mine, by using the microdissection needle, are identical in that one of the factors concerned in cell division is a cytoplasmic gelatinization.

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[8] Heilbrunn's paper giving his results in detail will appear with this paper, or shortly after.

The interior cytoplasm of a marine egg is a viscous fluid. The viscosity is high enough to prevent any Brownian movement of the enclosed granules but low enough to allow of their free movement when pushed about with the needle. The transition from the sol to a gel state is gradual. In the formation of the asters the gel state is rigid enough to hold the granules in a comparatively constant position since strands of the gel are dragged about with the needle. It is, however, much less rigid than solid gelatin which can be cut into discrete non-glutinous masses. It is also very easily reversible, for, in the early aster stage, a slight puncture and tear with the needle will cause it almost immediately to pass back into the sol state. The gel state here is never even visibly an inert solid for one is always conscious of a very gradual almost imperceptible movement and change. The picture of a gelled strand changes from minute to minute the difference being appreciable as time passes. Living protoplasm even in the gel state, is a dynamic structure never a static one.

IV. SUMMARY

1. The sphere is a liquid region free of granules, occupying the center of the aster and increasing steadily in size until the aster reaches full development.

2. The increase in size of the sphere is apparently due to the accumulation of liquid flowing into the sphere from all parts of

the cytoplasm. The aster rays appear to be the channels in which the centripetal flow occurs.

3. The cytoplasm between the rays is in the gel state to which the rigidity of the aster is due. The gel state is most pronounced near the sphere and peripherally passes gradually into the sol state of the cytoplasm lying beyond the confines of the aster. When the aster reaches the periphery of the cell the entire cell is rendered comparatively rigid.

4. In the maturation figures of the egg nucleus the peripheral aster forms a continuous gel with the surface layer of the egg to which the figure is thus firmly attached. The confines of the central aster pass insensibly into the surrounding liquid cytoplasm.

5. A periodic reversal of the sol to the gel state and vice versâ has been demonstrated in the cell protoplasm during division. The steps taken may be divided into the following series: (a) When the aster is fully formed the greater part of the cell is a gel. (b) The cytoplasm reverses to a sol state and the astral radiations fade out while the sphere liquid collects at the two poles of the nucleus. (c) The formation of radiations about the spheres at each pole of the nucleus producing the amphiaster is accompanied by a return to the gel state. (d) A return to the sol state takes place in the equator of the cell. The nuclear spindle now divides, a constriction around the middle of the cell then follows and continues until the cell is cut in two.

6. As a general rule, one may say that the reversal of the gel to the sol state starts in the equator of the cell and spreads to the poles. On the other hand, the reversal of the sol to the gel state commences about the sphere and spreads peripherad.

7. The gel state in living protoplasm is not inert. Even to the eye, there is always a constant but very gradual change among the granules embedded in the cytoplasmic gel.

8. There are appreciable differences in the liquid state of the cytoplasm in certain regions and at various times, e.g., the interior cytoplasm of the unfertilized and fertilized egg before the aster is formed is slightly viscous, whereas, the contents of the

sphere and of the rays, also the hyaline area in the vicinity of the forming polar body, are very fluid.

9. The aster in its early stage can be made to disappear by churning the cytoplasm with a needle. This causes a reversal of the gel to the sol state.

10. From a study of cell division in the eggs of Echinarachnius, Cerebratulus, Arbacia and Asterias, it may be concluded that one of the factors concerned lies in a peculiar colloidal property of protoplasm, viz., a periodic reversibility in its sol and gel states.

V. BIBLIOGRAPHY

Barber, M. A. 1914 The pipette method in the isolation of single microorganisms and in the inoculation of substances into living cells. The Philippine Jour. of Sc., vol. 9, Sec. B., Tropical Medicine, p. 307.

Bütschli, O. 1892 Investigations on microscopic foams and on protoplasm. Trans. by E. A. Minchin, 1894. London: A. and C. Black.

Chambers, Robert, Jr. 1915 Microdissection studies on the physical properties of protoplasm. Lancet-clinic, Mar. 27, 1915, Cincinnati.

1917 a Microdissection studies, I. The visible structure of cell protoplasm and death changes. Amer. Jour. Physiol., vol. 43, p. 1.

1917 b The microdissection method. Biol. Bull., vol. 32.

Coe, W. R. 1899 The maturation and fertilization of the egg of Cerebratulus. Zoöl. Jahrb., Bd. 12.

Conklin, E. G. 1902 Karyokinesis and cytokinesis in the maturation, fertilization and cleavage of Crepidula and other gastropods. Jour. Acad. Nat. Sci., Phila., vol. 12, ser. 2.

1905 The organization and cell lineage of the Ascidian egg. Jour. Acad. Nat. Sci., Phila., vol. 13, ser. 2.

1912 Experimental studies on nuclear and cell division in the eggs of Crepidula. Jour. Acad. Nat. Sci., Phila., vol. 15, ser. 2, p. 503.

Fischer, Alfred 1899 Fixierung, Färbung und Bau des Protoplasmas. Jena, G. Fischer, 362 pp.

Gaidukov, N. 1910 Dunkelfeld-Beleuchtung und Ultra-Mikroskopie in der Biologie und in der Medizin. Jena, G. Fischer, 84 pp.

Hardy, W. B. 1899 Structure of cell protoplasm. Jour. Physiol., vol. 24, p. 158.

Hartog, Marcus 1905 The dual force of the dividing cell. Part I. The achromatic spindle figure illustrated by magnetic chains of force. Proc. Roy. Soc., London, vol. 76.

Hatschek, Emil 1913 An introduction to the physics and chemistry of colloids. London: Churchill.

Heilbrunn, L. V. 1915 Studies in artificial parthenogenesis II. Physical changes in the egg of Arbacia. Biol. Bull., vol. 29, p. 149.

Heilbrunn, L. V. 1917 An experimental study of cell division. Abstract No. 14 in Proc. Amer. Soc. Zool., Anat. Rec., vol. 11, p. 487.

Kite, G. L. 1913 Studies on the physical properties of protoplasm. I. The physical properties of protoplasm of certain animal and plant cells. Amer. Jour. Physiol., vol. 32, p. 146.

Kite, G. L. and Chambers, R., Jr. 1912 Vital staining of chromosomes and the function and structure of the nucleus. Science, N. S., vol. 36, p. 639.

Liesegang, R. Protoplasma-Strukturen und deren Dynamik. Arch. Entwicklungsmech., Bd. 34, S. 452.

Lillie, F. R. 1909 Karyokinetic figures of centrifuged eggs; an experimental test of the center of force hypothesis. Biol. Bull., vol. 17, p. 101.

Marinesco, G. 1912 Forschungen über den kolloiden Bau der Nervenzellen und ihre erfahrungsgemässen Veränderungen. Koll. Ztschrft., Bd. 11, S. 209.

Mark, E. L. 1881 Maturation, fecundation and segmentation of Limax campestris, Binney. Bull. Mus. Comp. Zool., Harvard, vol. 4.

Mathews, A. P. 1906 A note on the structure of the living protoplasm of Echinarachnius eggs. Biol. Bull., vol. 11, p. 137.

Morgan, T. H. 1896 The production of artificial astrospheres. Arch. Entwicklungsmech., Bd. 3, S. 339.

1899 Action of salt solutions on the unfertilized and fertilized eggs of Arbacia and of other animals. Arch. Entwicklungsmech., Bd. 8, S. 448.

1908 The effects of centrifugal force on the eggs of Cumingia. Science, N. S., vol. 27, p. 446.

1910 Cytological studies on centrifuged eggs. Jour. Exp. Zoöl., vol. 9, p. 593.

Mott, F. W. 1912 The bio-physics and bio-chemistry of the neurone. Brit. Med. Jour., Sept. 28, p. 780.

Painter, T. S. 1916 Contributions to the study of cell mechanics. 1. Spiral asters. Jour. Exp. Zoöl., vol. 20, p. 509.

Price, S. Reginald 1914 Some studies on the structure of the plant cell by the method of darkground illumination. Ann. of Bot., vol. 28, p. 601.

Rhumbler, L. 1896 Versuch einer mechanischen Erklärung der indirekten Zell und Kerntheilung. Arch. Entwicklungsmech., Bd. 3.

1898 Referat über allgemeine Zell mechanik. Ergebn. Anat. u. Entwicklungsgesch., Merkel Bonnet, Bd. 8, S. 543.

Spooner, G. B. 1911 Embryological studies with the centrifuge. Jour. Exp. Zool., vol. 10, p. 23.

Wilson, E. B. 1895 Archoplasm, centrosome and chromatin in the sea-urchin egg. Jour. Morph., vol. 11, p. 443.

1900 The cell in development and inheritance. 2nd ed. Columbia Univ. Ser., vol. 4. The MacMillan Co., 1904.

1901 a Experimental studies in cytology. 1. A cytological study of artificial parthenogenesis in sea-urchin eggs. Arch. Entwicklungsmech., Bd. 12, S. 529.

1901 b Experimental studies in cytology. II. Some phenomena of fertilization and cell division in etherized eggs. Arch. Entwicklungsmech., Bd. 13, S. 353.

WILSON, E. B., and MATHEWS, A. P. 1895 Maturation, fertilization, and polarity in the echinoderm eggs. Jour. Morph., vol. 10 pp. 319.

YATSU, N. 1905 The formation of centrosomes in enucleated egg-fragments. Jour. Exp. Zoöl., vol. 2, p. 287.

ZSIGMONDY, R. 1905 Zur Erkenntnis der Kolloide. Jena: G. Fischer. Trans. by J. Alexander. New York: Wiley.

PLATE I

EXPLANATION OF FIGURES

The figures are all sketches from living eggs. Figures 1 to 13 are of the Echinarachnius egg, figures 14 to 20 of Cerebratulus. Figures 1, 2, 10, 14, 15 and 17 to 20 purport to be more or less faithful representations. The remainder are diagrams in which the astral radiations are merely indicated by lines.

The tip of the needle when shown is represented by a small circle with a central spot.

1 Enlarged drawing of the tapering tips of the gelled cytoplasm with embedded micro- and macrosomes) projecting between bases of the hyaline liquid rays into the periphery of the sphere.

2 The same as figure 1 but with the tips bent to one side by the microdissection needle.

3 Diagram to show the distortion of astral radiations by inserting the needle into the aster and moving the needle in a straight line.

4 Nucleus in a fully formed aster pushed to one side with a needle thus causing distortion and stretching of the astral radiations.

5 Early sperm aster spirally twisted in shape by moving it through the liquid cytoplasm with a needle.

6 Inserting the needle into the sperm nucleus and pulling draws the nucleus out into a ribbon-shaped mass showing that the nucleus is held in a gelatinous mass. On being released the nucleus reverts to its original spherical shape.

7 Fusion nucleus lying to one side in the sphere so that the sphere-liquid extends like a cap around one side of the nucleus.

8 Nuclear 'spindle' becoming bipramidal in shape as the sphere-liquid begins to collect at its two poles. Rays beginning to disappear around the equator.

9 Hyaline plasma collected at the nuclear poles to form two spheres. Beginning of the amphiaster.

10 Amphiaster in living egg of the Echinarachnius. The drawing shows only the central portion of the amphiaster. The clear area extending between the two asters represents the hyaline nuclear 'spindle.' The chromosomes have been omitted in the drawing. Scattered microsomes mark off the ends of the 'spindle' from the sphere.

11 Nuclear 'spindle' beginning to take on the form of a dumb-bell. The cytoplasmic granules in the equatorial region about the nuclear constriction become visibly mobile at this stage indicating an increase in the fluidity of that region.

12 The needle inserted into the nuclear 'spindle' has pulled it to one side in the cell dragging the astral radiations along with it.

13 Egg cleavage nearly completed. The nuclear body has separated into the two daughter nuclei. Rays are absent in the equatorial region which is probably in the sol state. The sphere-liquid extends over part of each daughter nucleus like a cap.

14 Hyaline area in Cerebratulus egg with the egg nucleus preparatory to the formation of the first maturation figure.

15 First maturation spindle with amphiaster.

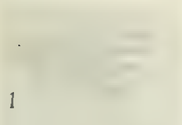
16 First maturation anaphase 'spindle' stretched by the needle in an attempt to pull it away from the egg periphery. The peripheral aster and the egg surface form a continuous gel thus anchoring the 'spindle.'

17 First polar body almost completely extruded. The egg nucleus lies in a clear area below the polar body.

18 Interkinetic egg nucleus pushed out of its normal position into the granular cytoplasm.

19 Granules in the cytoplasm receding from the dislocated egg nucleus so that it is soon surrounded by a clear area again. The area originally occupied by the nucleus is being invaded by granules so that in time it cannot be distinguished from the general cytoplasm.

20 Second polar body formed by the dislocated egg nucleus on the egg periphery some distance from the first.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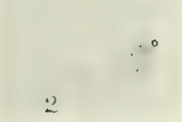
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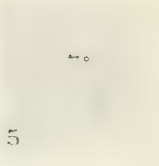
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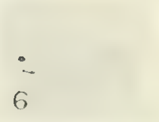
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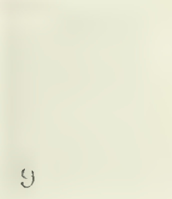
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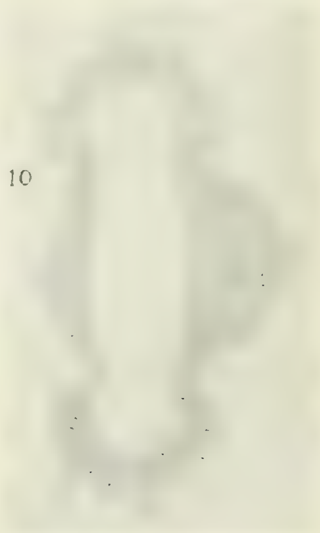
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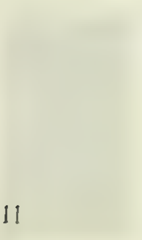
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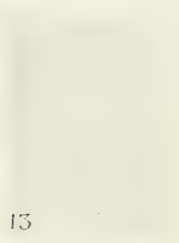
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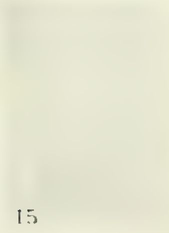
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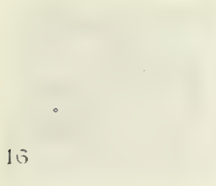
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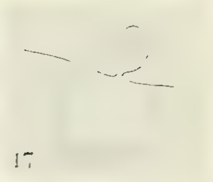
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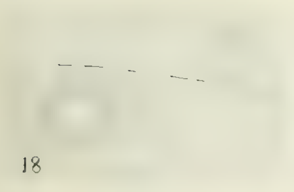
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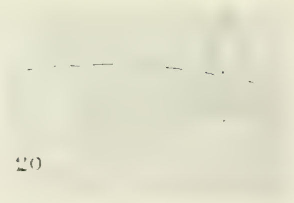
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January, 1917

*15. *Microdissection studies.* Cell and nuclear division. (Lantern).

ROBERT CHAMBERS, Jr., Cornell Medical College, New York City.

Cortical changes in the egg cell on the approach of cell division can be demonstrated with the needle. In polar body formation the change appears to be due to the presence of the nucleus for, on removal of the nucleus to another region of the cell, a corresponding change in the cortex occurs. Preparatory to division the bulk of the cytoplasm undergoes temporary gelation and constriction takes place in the region where liquefaction sets in.

The resting nucleus is an optically homogeneous body in which a granular network is often produced on the slightest injury. It is fluid in consistency in egg cells and possibly in other cells. Formation of the nuclear spindle was studied in germ cells and in egg cells during polar body formation and segmentation. In no case could spindle fibers be demonstrated. The nuclear substance assumes a spindle shape under the influence of the centrospheres. Liquefaction of the cytoplasm in the equatorial region of the cell is accompanied by a constriction which shapes the nuclear spindle into a dumb bell during the ana-and telo-phases. As the daughter nuclei draw away from one another they are connected by a strand which is of a sufficient consistency to distort the nuclei if caught and pulled by the needle.

The prechromosomal filaments appear in the prophase out of the hyaline nuclear matrix through the precipitation of granules in more or less irregular masses closely investing long slender cylindrical strands of a gel like consistency. Many of the diplotene filaments figured in fixed material may posssibly be due to the collapse of these strands into ribbon like structures with an accumulation of the granules along the edges. Shortening of the prechromosomal filaments is accompanied by a fusion of the granules thus forming the definite hyaline, viscous and comparatively rigid chromosomes. The chromosomes are imbedded in the nuclear spindle mass and undergo movements resembling those in drops which are undergoing changes in surface tension. The constriction of the nuclear spindle at its middle possibly aids the migration of the chromosomes by pushing them to the poles.

Many if not all of the structures which appear during cell and nuclear division may be explained as reactions in a mixture of colloidal materials producing local liquefactions together with precipitations and gelatins which may be made to disappear on changing the reaction.

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THE MICROVIVISECTION METHOD.

ROBERT CHAMBERS,

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The dissection of living cells has hitherto proved inadequate for a study of the physical properties of protoplasm and of its structural components. This is owing largely to a lack of means for the manipulation of dissecting instruments under a sufficiently high magnification of the microscope.

Before and since Barber first devised his mechanical pipette-holder for the isolation of bacteria, various ingenious methods for the dissection of microscopic objects have been described. They all, however, fall short of Barber's method, because, with them, only comparatively low powers can be used, since the dissecting needles must operate *between* the objective and the tissue to be dissected. On the other hand, by using needles instead of micro-pipettes, Barber's instrument has been converted into an excellent microdissection apparatus. The needles operate in a shallow hanging drop containing the cells to be dissected which are pressed against the *undersurface of a coverslip* in a moist chamber. There being no obstacle above the coverslip, oil immersion lenses may be used for observation.

In an article appearing as long ago as 1859, Dr. H. D. Schmidt, of Philadelphia, describes in detail an excellent "microscopic dissector" consisting of a base to be fastened on the stage of a microscope with a number of clamps to hold instruments, each clamp possessing three movements controlled by screws. A lever fastened in one of the clamps holds the tissue in place. Fine scissors, knives or steel needles are fastened in the other clamps. By turning the various screws, the instruments can be brought into place and be operated with remarkable accuracy. Dr. Schmidt worked with the tissue, the instruments and the lower lens of the objective immersed in water or diluted alcohol. The full results of his investigations with this instrument were not published until 1869, owing to interruptions due to the Civil

War, during which his drawings and manuscript were burned in a conflagration in Washington. In his later paper he has two illustrations showing a liver cell being torn in two by needles.

In 1887 Chabry described an apparatus in which a glass needle was held in a sheath in such a way as to allow the needle to be pushed to any prescribed distance into the bore of a glass capillary tube. The bore of the capillary was made just large enough to admit a single ovum and hold it in place. By proper adjustments, the needle could be pushed to any depth in the ovum. The apparatus was placed on the stage of a microscope and Chabry was able to injure locally a *Strongylocentrotus* ovum while under observation. Chabry's instrument has been used for experimental embryological work and is described in detail in Ehrlich's "Enzyklopädie für microskopischen Technik," 1910.

In 1907 J. F. McClendon first described his "mechanical finger," which consisted of an ordinary Spencer mechanical stage with an additional screw to allow of movements in three different directions and a clamp to hold a needle or pipette. With this apparatus, McClendon was able to suck the nucleus out of a *Chætopterus* egg. In 1909 he described an improved form with which he dissected certain Protozoa.

In 1912 an apparatus was described by Tschachotin. It consists of a clamp attached to the side of the objective of a microscope. In the clamp is fastened a needle which curves so as to project under the objective and is so adjusted that the tip lies in the focus of the objective. The needle point is lowered into the object to be dissected simply by bringing the object into view under the microscope and lateral tears are made on moving the object with the slide by means of the mechanical stage.

In 1904 Barber first described his method of using a hanging-drop in a moist chamber for isolating microörganisms. A fuller account appeared in 1907 in which he first describes a mechanism for holding micropipettes and where he mentions Montrose T. Burrows as having assisted him much in its design. Elaborations of his instrument appeared in his papers of 1908 and 1911. In 1911 he suggested the possibility of the use of his instrument for cell dissection and for investigation on fertilization and heredity problems. His paper of 1914 is a detailed and precise description of his technique and is of the utmost value for the prospective worker in micro-dissection. As he developed his method principally for the use of pipettes and also because his latest paper appeared in a publication not readily obtainable, I have endeavored to give here a description of the method with modifications and new developments in the application of the method to microvivisection.[1]

A simple form of Barber's apparatus is shown in Fig. 1. The moist chamber, which is open at one end and with sides from 8 to

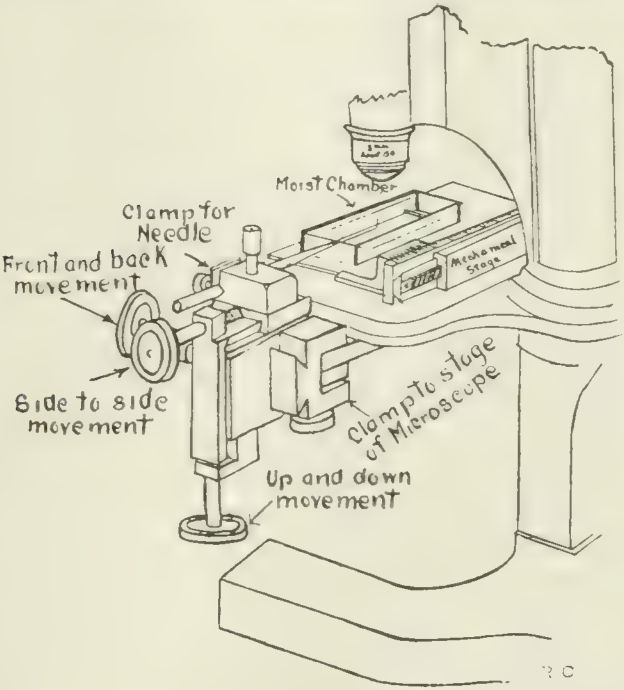

FIG. 1. Barber's single three-movement pipette holder, glass needle and moist chamber arranged to illustrate method of dissecting cells in a hanging drop under the highest magnification of the microscope. (Substage of miscroscope omitted in drawing.)

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[1] In 1912, G. L. Kite first applied Barber's apparatus to microdissection, which quickly resulted in the publication of several papers. Kite was to have prepared an article on the method when his health unfortunately broke down. Publications on microdissection have appeared as follows: 1912, Kite (on the fertilization membrane) in *Science*, N. S., XXXVI.; Kite and Chambers (on chromosomes and nucleus) in *Science*, N. S., XXXVI. 1913, Kite (on permeability of cells) in BIOL.

12 mm. high, is placed on the microscope so that it may be moved about with the mechanical stage. The chamber is roofed over with a carefully cleaned coverslip, on the under surface of which, the specimen is mounted in a shallow hanging drop of a physiologically indifferent fluid. The dissecting needle is made by drawing out one end of a piece of glass tubing and bending it at right angles two or three millimeters from the pointed tip. The needle-holder, a mechanism allowing of three movements, is clamped to one side of the microscope stage and the needle is adjusted so that it may project into the moist chamber with its tip pointing up into the hanging drop. By proper adjustment the cell to be dissected and the point of the needle are brought into the same focal field. The three movements of the needle, permitted by the needle-holder, and the two movements of the moist chamber, by means of the mechanical stage, give the experimenter ample opportunity to carry on dissection under the highest magnification of the microscope.

The Instrument.

The holder accommodated to carry only one pipette or needle is illustrated in Fig. 1. It is useful in many ways and is comparatively inexpensive. Its disadvantage lies in the fact that the resistance of the cell-substance to the needle may overcome the inertia of the cell, so that, instead of tearing through the cell, the needle simply drags it about. However, by careful manipulation and by the possible use of a viscous dissection-medium much can be done. Many marine ova and germ cells in general are very soft in consistency and, if the drop in which they are immersed be shallow enough, the surface tension of the drop flattens them against the coverslip and is sufficient to hold the cell. The single holder can also be successfully used for injecting material into or extracting material out of a cell. In addition to the movement of the needle, manipulation is greatly aided by the

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Bull., XXV., and (on protoplasm) in *Amer. Jour. Physiol.*, XXXII., 1914, Chambers (on the nucleus) in *Science*, XL.; Kite (structural transformations of blood cells) in *Jour. Inf. Dis.*, XV. 1915, Chambers (on the germ cell) in *Science*, XLI.; and (on protoplasm) in *Lancet-Clinic*, March 27, Cincinnati; Kite (on cell permeability) in *Amer. Jour. Physiol.*, XXXVII. 1917, Chambers (on protoplasm) in *Amer. Jour. Physiol.*, XLIII.; and (on the cell aster) in *Jour. Exp. Zoöl.*, XXIII.

use of the mechanical stage by means of which the moist chamber may be moved in two directions.

The double holder, formerly manufactured by the Fowler shops of the University of Texas, is twice as expensive as the single holder. It possesses, however, the advantage that two needles or pipettes may be used simultaneously. Each needle can be moved independently of the other and either needle may be replaced by a pipette. The manufacture of the holder has recently been duplicated by the mechanician of Wesleyan University under the direction of Dr. H. B. Goodrich with modifications of his and my suggestion. In setting up the apparatus, Dr. Goodrich introduced the useful innovation of having the shelf to which the instrument is clamped on a stand independent of the microscope. The two stands are then clamped together on a common base. The operator can thus shift the position of the instrument placing it at will in front of or on either side of the microscope.

Fig. 2 illustrates the double holder as I have it set up. The attachment of the instrument to the front of the microscope stage facilitates manipulation of the screws with both hands. In the illustration, a Spencer Lens Company lamp replaces the substage mirror. As the heat of the lamp, however, produces undue evaporation and subsequent condensation in the moist chamber, it is preferable to use the mirror. This necessitates raising the microscope-stand on a block of wood so that the mirror can be lowered below the microscope base so as to receive light unobstructed by the lower screws of the dissecting instrument.

The needle-carrier with its groove (Fig. 2, *a*), in which the arm of the needle lies, is twice as long as in Barber's original instrument. This extra length assists one in giving a straight even movement to the needle when it is being pushed into the moist chamber. After the needle has been thus adjusted by hand approximate to its proper position, the plate *b* is pushed over the arm of the needle and the screw *c* tightened to clamp the needle. Further adjustment of the needle is carried out by the screws *e*, *f*, *g* and *h* under the low and high powers of the microscope.

In preparing the shelf on which the instrument is to be clamped (Fig. 2, *j*) care must be taken that it be low enough to bring the top of the carrier flush with the upper surface of the microscope

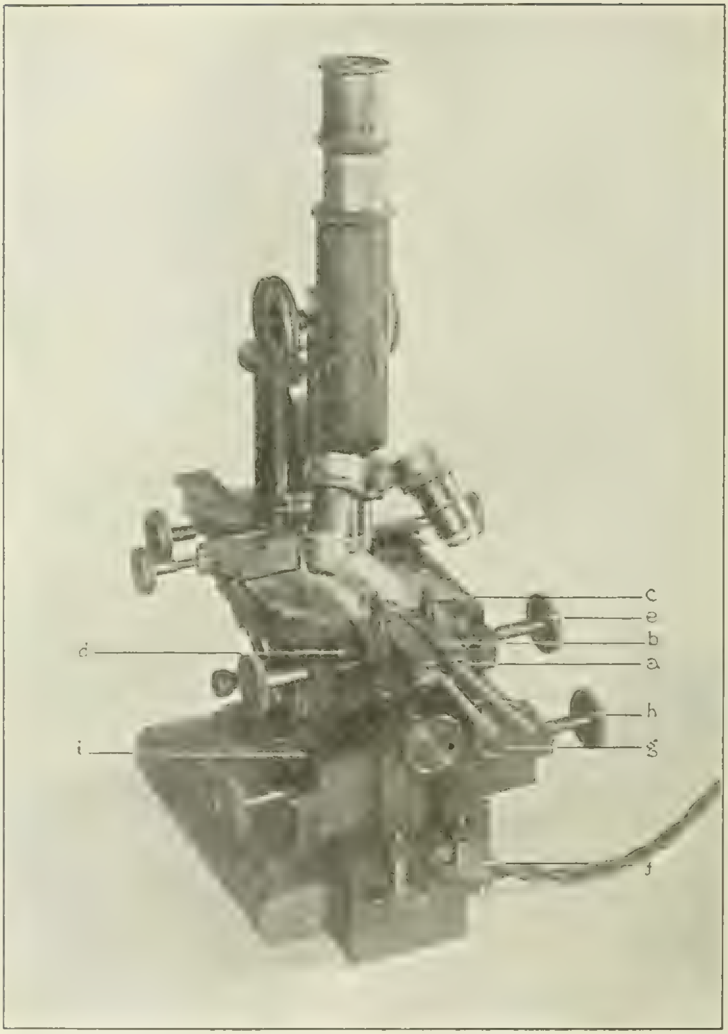

FIG. 2. Double pipette holder set up for microdissection. *a*, Carrier with groove to hold needle. *b*, brass plate which slips sideways over the groove in which needle lies. (The undersurface of the plate is covered with blotting paper to give it resiliency where it comes into contact with the glass needle.) *c*, set screw to clamp plate on carrier. *d*, screw which, on loosening, allows carrier to be set at any angle. *e–h*, screws to move the needle (*e*—side to side, *f*—up and down, *g*—in and out, *h*—side to side for both carriers together). *j*, shelf to which instrument is clamped.

stage. This is in order to enable one to use a moist chamber of the minimum height (see below).

An important factor in the manufacture of the microdissection instrument is that the two horizontal movements run true and keep the needle point always in the same plane. Otherwise, at a critical moment in the dissection, a reversal of a horizontal screw may either suddenly lower the needle-point out of focus or jam it against the coverslip and break it. Unfortunately, the last model put out by the University of Kansas is deficient in this respect. The existence of lost motion in the screws can be taken care of in the manipulation but if the needle tip does not run true in a horizontal plane the peace of mind of the operator may be sorely tried!

THE MOIST CHAMBER.

The moist chamber (Fig. 3) is a glass slide to which are cemented strips of glass in such a way as to form a box open at the top and at one end. For cement Canada balsam may be used.

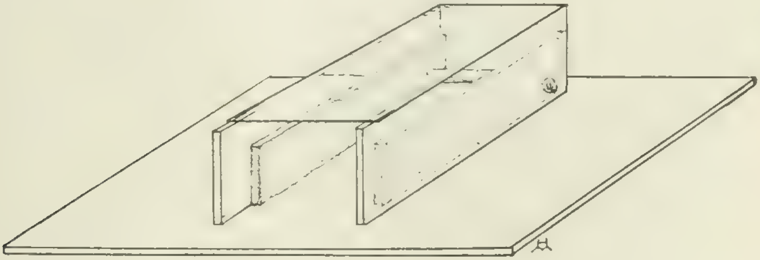

FIG. 3. Moist chamber for microdissection.

A shallow trough, to hold water, is made with a narrow glass strip set across the floor of the chamber 8 or 9 mm. from the closed end. This and other small strips set outside the chamber serve to reinforce the walls. Care must be taken that the upper edges of the chamber be even and level when placed on the stage of the microscope. A carefully grease-free cleaned coverslip with a hanging drop containing the objects to be dissected forms the roof of the chamber. The roof is sealed on with vaseline smeared along the upper edges of the walls of the chamber.

The height of the chamber is conditioned by the focal distance of the condensor used and by the minimum working room that one must have in which to manipulate the needles. For routine work it may be well to have a chamber of fairly large dimensions disregarding the condensor as it is surprising how much illumination a condensor will give beyond its focal distance. However, for critical work, it is imperative to have the height of the chamber such that the objects on the undersurface of the coverslip be close to the focus of the condensor. My condensor has a working focal distance of almost 9 mm. and the chambers I use are 45 mm. long, 22 mm. wide and 9 mm. high.

The moist chamber is lined on the sides with blotting paper leading from the trough to carry the water along the length of the chamber so as to furnish a large moist surface. The liquid in the trough and the blotting paper should be isosmotic with the liquid used for the hanging drop so that evaporation be equalized and the hanging drop not become diluted.

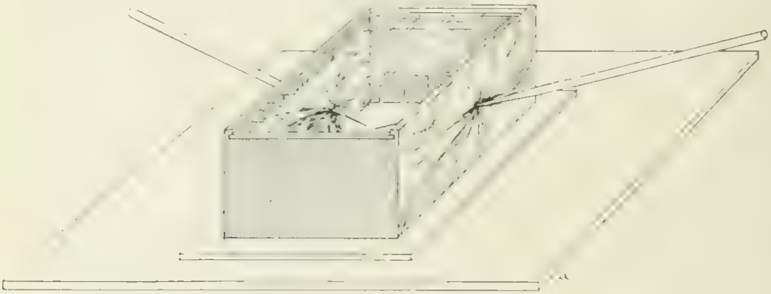

FIG. 4. Moist chamber for preliminary teasing of tissue.

For the dissection of the cellular elements of somatic tissues, it is necessary to do some preliminary teasing under an ordinary dissecting microscope. This should be done in a moist chamber of some kind. The tissue is teased on a coverslip which is then inverted and placed on the microdissection moist chamber.

A handy moist chamber (Fig. 4) can be made out of a shallow box with dimensions approximately 7 × 5 × 3 cm., with a top and bottom of glass and the two longer sides of thin rubber sheeting. The coverslip with the tissue to be teased is placed within the box and the teasing is carried out with needles projecting through the rubber sheeting. For the tissue of warm-blooded animals both the preliminary teasing and the microdissection should, of course, be carried on in warm boxes.

Tissue cultures are grown on square coverslips. The slips are then placed on the moist chamber for microdissection, the parts of the chamber not roofed over being previously covered with slips of glass.

THE NEEDLES.

These are made from glass tubing with an outside diameter of about 4–6 mm. and a lumen of 3–5 mm. The technic is readily mastered with soft glass tubing but needles made of hard glass are more durable.

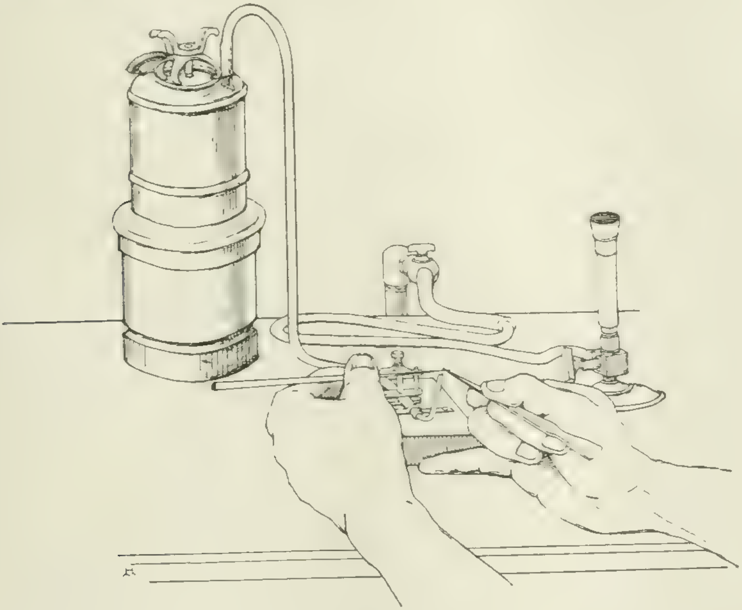

FIG. 5. Method of making the capillary pipettes and microdissection needles.

The needle points are made over a minute flame. A serviceable micro-burner with an acetylene generator is shown in Fig. 5. The microburner is made out of a piece of hard glass tubing bent almost at right angles and with one end closed except for the smallest possible aperture that will retain a flame. This is done by heating the end and pinching it with a pair of forceps. When set up the tip of the burner should be at a height of about 5–6 cm. above the surface of the table. The size of the flame is regulated by the screw pinch-cock set on the rubber tube connecting the burner with the gas generator.

As a source for the flame acetylene is to be preferred to ordinary illuminating gas because with the former a narrower flame can be obtained without undue clogging. The acetylene generator in the figure is part of an old bicycle lamp. A more convenient way is to have the acetylene in a small compression tank which can be recharged at a very small cost. If, however, ordinary gas is to be used, one may much improve the gas by passing it through alcohol or benzene.

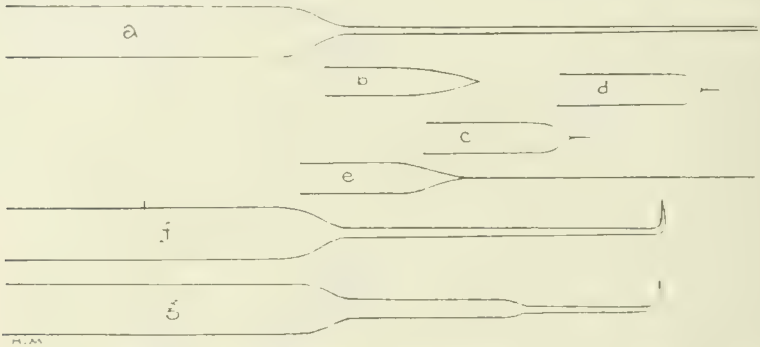

FIG. 6. Stages in making needles. *a*, glass tube with one end drawn out into a capillary. *b*, good tapering point. *c* and *d*, serviceable points. *e*, tip drawn out into a hair. *f*, completed microdissection needle. *g*, needle made on a relatively stout shank.

The method of procedure in making the glass needle is as follows:

1. In an ordinary Bunsen burner (or a Mecca burner if hard glass be used) draw out one end of a piece of glass tubing into a straight capillary about 0.3–0.5 mm. in outside diameter (Fig. 6, *a*). Prepare a supply of such pieces with the capillary ends at least 6 cm. long.

2. Lower the flame of the microburner to the smallest flame that will remain lighted. The microburner should be protected from draughts of air and in semi-darkness so that the flame may show up to best advantage.

3. Hold the shank of the glass tube in the left hand and, with a pair of fine forceps held in the right, grasp the capillary at a point about 5 cm. from the shank. Bring the portion of the capillary next the forceps over the flame and at right angles to it at a point just over the flame (Fig. 5). Pull gently with the

forceps and, when the glass begins to soften, lift it slowly from the flame and pull with the forceps slightly more than at first, but not too strongly. The hands should remain on the table during the process and the pulling and lifting done by turning them slightly outward. The capillary will separate with a slight tug—a feeling much like that experienced when a taut thread, held in the fingers, is parted in a small flame. If the point is properly made, it will appear as shown in Fig. 6, *b*, *c* and *d*. This capillary has sufficient rigidity and it comes to a very fine point. The tip is closed, but the lumen extends almost to the end. It is evident that everything depends upon the amount of heat used and the timing of the pull, and that these must vary slightly with the height of the flame and the diameter of the capillary. With a little experience, one can usually tell when a proper point is made by the peculiar feeling described above. If too little heat is used and the pull made too suddenly, the capillary may part with a snap and the tip be found broken off short often forming a serviceable pipette. If too much heat is used, the capillary is apt to be drawn into a long hair (Fig. 6, *e*).

4. After a suitable point is made, the end of the capillary is turned up at right angles (Fig. 6, *f*). This is done by holding the point of the capillary just back of the point above the small flame and pushing up the end with the tip of the forceps or with a needle. Not more than 5 mm. should be turned if it is to be used in a chamber not over 8 mm. in height. If a good point is made, *e. g.*, Fig. 6, *b*, it is not necessary to turn the end at exactly right angles. A pipette is made by jamming the tip of a needle against the cover glass in the moist chamber to break off the point.

The forceps used in the making of the needles should be of a size that can be firmly grasped in the hand. The fine tips should be accurately set and almost parallel one another for a few millimeters. In order to give the tips a good, grasping, resilient surface I dip them, before using, in a small vial of Sandarac varnish which may be obtained from a dentists' supply dealer. As the varnish burns if brought too close to the flame, it is well to use a second forceps or a needle for bending the glass capillary.

Fine points are not readily made from capillaries of a diameter greater than .05 mm. Therefore, if the dissection requires a comparatively stiff shank for the dissecting needle, start with a glass tube possessing a thick capillary and draw out the tip into a thinner capillary. This thinner capillary may then be used for making the needle point (Fig. 6, *g*).

The facility in making the needle-points over the microburner increases, of course, with practice. In my case, I make at times twenty or thirty within an hour, at other times none, or only one or two at the most. A supply may be kept on hand on a stand made of two horizontal layers of wire netting, the meshes of which admit the base of the needles. It is well to mark off the stand into compartments to receive the various types of needles that are made.

Another good method for making the needle points is that of Chabry ('87). The tip of a capillary pipette is brought into contact with a heated mass of glass (or any incandescent body to which glass will adhere) and suddenly drawn away. For an incandescent body Chabry used the blade of a platinum knife of a surgical instrument for thermocautery. The glass capillary was held by the operator in a groove on a stand a few centimeters from the platinum blade, which was fastened so as to be immovable. An assistant controlled the heating of the platinum blade. As soon as it heated to a dull glow, the operator slid the capillary on its groove until the tip touched the glowing metal when it was instantly slid back. The sliding of the capillary in a stationary groove insures a tapering to a point in a straight line with the long axis of the capillary. In his paper Chabry discusses this method in some detail, pointing out ways of avoiding difficulties in the technique ('87, pp. 175–178). Chabry also suggested the use of insect mouth parts, annelidan bristles and the spicules of sponges for the tips of microdissection needles. Barber suggested fine-pointed needle crystals, or the sharp, stiff hairs taken from the body of a house fly. A pipette with a fairly large opening is made and the hair or crystal is drawn partially into it. The fine point projecting from the tip of the pipette is then used as a probe or dissecting needle. Such needles could be used only on very delicate soft objects.

Needle points may also be made by grinding one end of a fine wire. To produce chemical effects in a cell, McClendon ('09) used a copper wire ground to a point and further sharpened by erosion in acid. The chemical injury produced by metal needles limit their use for the dissection of living cells.

For experiments in cutting cells, one must take into account the fact that many cells will lose their more or less fluid contents if a gap be torn in their surface. If, however, the side of a fine needle can be brought to press the cell against the coverslip, a constriction will result which may cut the cell into two intact pieces. For cutting soft-bodied ova and protozoa this can be done with a form of needle depicted in Fig. 7. The needle tip is bent back rather than forward to lessen the chances of breaking the tip when adjusting the needle in the moist chamber and also when the needle needs to be cleaned (see below). The tip should not point directly back but slant off to one side (see Fig. 7, *b*), so that the shank of the capillary will not be in the way of the light coming up from below. The angle at which the needle is bent must be ascertained by successive trials, for, if it be bent too near to a horizontal plane, the tapering tip will bend away from the cell instead of cutting into it. If the angle be too far from the horizontal plane, the cell will slip along the needle and escape. When the proper angle is obtained, one can cut *Arbacia* and *Asterias* ova into pieces of almost any required size with considerable ease. A clean cut through an unfertilized sea-urchin egg or a fertilized egg, before the fertilization membrane has become too tough, can be best made by bringing the egg between the horizontal arm of the needle and the lower surface of the hanging drop (Fig. 8). On lowering the needle out of the drop,

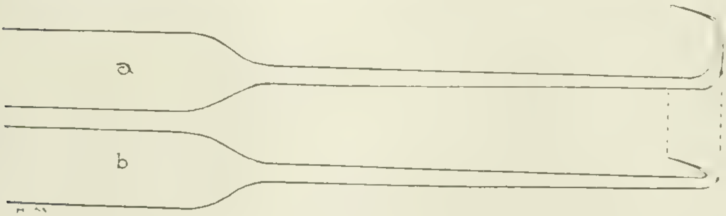

FIG. 7. Needle with tip curved back to almost an horizontal plane. *a*, lateral view. *b*, view from above.

the arm of the needle passes through the egg cutting it cleanly in two.

A good needle, when once made and proven to be resilient and strong, may be used repeatedly. Mucinous material which soon covers its tip so as to render it useless can be got rid of by immersing the needle successively in a strong acid and a strong alkali.

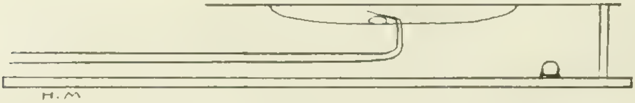

FIG. 8. Side view of moist chamber to show method of cutting an egg cell in two with needle shown in Fig. 7.

CELL INJECTION.

Barber, in his two papers ('11 and '14) gives a full description of his admirable mercury method for injection. With it one can inject microscopically minute doses of a solution into any part of a cell. The advantage of the pressure produced by the mercury over that by air lies in the slow driving force of expanding mercury which can be almost instantly arrested when the dose injected is considered sufficient. On the other hand, if air pressure be used, the air in the injection tube will compress enormously as the resistance of the bore at the tip of the pipette is too great to overcome. If the injection does occur it may go with a rush usually followed by a volume of air which destroys the cell being experimented upon. Notwithstanding this, it is surprising what delicate work may be done simply with air pressure. An example of this is the extraction of the nucleus from a mature *Arbacia* egg and its subsequent injection into another egg. For the injection of substances, however, where the amount must be strictly controlled, the mercury method is essential.

The extraction of material out of a cell is comparatively easy to carry out, as the capillary attraction in the lumen of a micropipette (the inside diameter of which may be anywhere from 2 micra up) is quite sufficient for the purpose. For injection purposes the difficulty lies in securing the force to overcome this capillary attraction.

Source of Light and Lenses.

A most serviceable light for microscopic work is the 100-watt horseshoe filament, Edison, Mazda, nitrogen lamp with the blue daylight glass. This lamp, when set in a frosted milk-white glass globe, gives a white light very restful to the eye.

In selecting lenses care should be taken to procure those possessing the maximum focal length together with the greatest numerical aperture. For this reason the Zeiss apochromatic 4 mm. dry lens with a N. A. 0.95 and the 3 mm. oil immersion lens with N. A. 1.40 are excellent for microdissection or for the study of living tissue in general. It is to be remembered that the equivalent focus of a lens does not correspond with the working focal distance.

A good substage condensor with a working focal distance of 8–9 mm. can be made by removing the top lens of the Leitz triple-lens, centering condensor. The beam of light issuing from it completely fills the back lens of a 4 mm. objective with N. A. 0.65 and more than half of a 1/12 oil immersion objective with a N. A. 1.30. This gives enough definition for such detailed cytological work as the study of the chromomeres in grasshopper germ-cells and of the types of protoplasmic granules in marine ova. One may obtain very good condensors with a long working focal distance from those used in projection apparatus where a cooling trough is inserted between the condensor and the microscope stage.

Setting up the Instrument.

The instrument is first clamped to the microscope and the moist chamber, with the edges of its walls smeared with vaseline, placed in the mechanical stage. The needle is then adjusted as follows: Place the arm of the needle in the groove of the carrier with the dissecting tip extending into the moist chamber. Push the plate of the carrier (*b*, in Fig. 2, page 126) over the arm of the needle and tighten the set screw *c* just enough to allow one to push the needle with a straight and even movement. The needle is now adjusted by hand so as to bring the tip within the field of a low power objective. The set screw is then tightened to clamp the needle in place. Further centering of the needle is made by the screws *e*, *f*, *g* and *h*, under the low and high powers of the microscope. Finding the tip of the needle under high powers is facilitated by using an ocular containing cross hairs.

The coverslip with its drop containing the tissue to be dissected is now inverted over the moist chamber. The cells to be dissected are brought into the field of the microscope. The tip of the needle is then raised by the screw *c* until brought into view first under low and then under high power. The apparatus is now ready for dissection.

In conclusion I wish to suggest to the prospective worker in this field that he use the utmost conservatism in his interpretations of observed effects produced in microdissection. One must constantly keep in mind the extraordinary instability of living cell structures.

BIBLIOGRAPHY.

Barber, M. A.

'04 A New Method of Isolating Microörganisms. Jour. Kans. Med. Soc., Vol. 4, p. 487.

'07 On Heredity in Certain Microörganisms. Kans. Univ. Sci. Bull., Vol. 4, p. 3.

'08 The Rate of Multiplication of *Bacilli coli* at Different Temperatures. Jour. Inf. Dis., Vol. 5, p. 379.

'11 A Technic for the Inoculation of Bacteria and other Substances and of Microörganisms into the Cavity of the Living Cell. Jour. Inf. Dis., Vol. 8, p. 348.

'14 The Pipette Method in the Isolation of Single Microörganisms and in the Inoculation of Substances into Living Cells. The Philippine Jour. Sc., Sec. B, Trop. Med., Vol. 9, p. 307 (reviewed in Zeitschr. wiss. Mikr., Bd. 32, S. 82, 1915).

Chabry, L.

'87 Contribution a l'embryologie normal et teratologique des Ascidies simples. Jour. de l'Anat. et de Physiol., Vol. 23, p. 167.

McClendon, J. F.

'07 Experiments on the Eggs of Chætopterus and Asterias in which the Chromatin was Removed. BIOL. BULL., Vol. 12, p. 141.

'08 The Segmentation of Eggs of *Asterias forbesii* Deprived of Chromatin. Arch. Entwicklungsmech., Bd. 26, S. 662.

'09 Protozoön Studies. Jour. Exp. Zoöl., Vol. 6, p. 265.

Schmidt, H. D.

'59 On the Minute Structure of the Hepatic Lobules Particularly with Reference to the Relationship between the Capillary Bloodvessels, the Hepatic Cells and the Canals which Carry off the Secretion of the Latter. The Amer. Jour. of the Med. Sci., N. S., Vol. 37 (Jan.), p. 2.

Tschachotin, S.

'12 Eine Mikrooperationsvorrichtung. Zeitschr. wiss. Mikr., Bd. 29, S. 188.

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A REVIEW OF THE CHROMOSOME NUMBERS IN THE METAZOA

PART I

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The following tabulation comprises the chromosome work that has been done since the pioneer researches of Van Beneden in 1883 until the end of 1915. The list is as complete as possible, and recourse has been had in all cases to the original publications. All the authorities have been given for each species, even when the accounts are conflicting, and these are given in the order of priority. All the species are classified and the various phyla, genera, families and species are arranged in alphabetical order. The present paper includes the Annelida, Arthropoda and Coelenterata, and will be followed by a second paper including a tabulation of the other invertebrates (excluding Protozoa) and the vertebrates together with some general remarks. Some of the most important abbreviations are:

chrom = chromosome
cl = cleavage
div = division
el = elements
fert = fertilized
mat = maturation
oog = oogonia
pa = parthenogenetic
p.b. = polar body
prim = primordial
pron = pronucleus
som = somatic
spc = spermatocyte
spg = spermatogonia

"X to pole" means that X passes undivided to one pole.

"XY to poles" means that X and Y pass to opposite poles.

"From correction in" means that the tabulation as given is a correction of an earlier account.

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
A. INVERTEBRATA **I. ANNELIDA** *a.* ARCHIANNELIDA							
Dinophilus gyrociliatus..	20 cl ♂ 20 cl ♀	20 ♂ egg (= 10 double) 20 ♀ egg (= 10 double)	10 ♂ egg (double)	10 ♂ egg (double)		Shearer, '11 Shearer, '12	Jour. Marine Biol. Assoc., 9, p. 156 Q. J. M. S., 57, p. 329
Histriobdella homari....		8 ♀				Shearer, '10	Q. J. M. S., 55, p. 237
Protodrilus purpureus ..		4 ♀	4 ♀	4 ♀		Pierantoni, '08	Flora u. Fauna Golfes Neapel, 31, p. 1
b. CHAETOPODA *1. Oligochaeta*							
Allolobophora foetida...	22 oog	11 ♀	11 ♀	11 ♀		Foot, '08 Foot and Strobell, '05 Foot and Strobell, '10	Jour. Morph., 14, p. 481 Amer. Jour. Anat., 4, p. 199 Arch. Zellf., 5, p. 149
Enchytraeus adriaticus .	24 oog					Vejdovský, '07	Kgl. böhm. Ges. Wiss. Prag, '07, p. 1
Enchytraeus humicultor .	32 spg 32 oog	16 ♀				Vejdovský, '07	Kgl. bohm. Ges. Wiss. Prag, '07, p. 1
Fridericia hegemon	64 spg 64 oog	32 ♀				Vejdovský, '07	Kgl. böhm. Ges. Wiss. Prag, '07, p. 1
Ilyodrilus coccineus......		16 ♀				Vejdovský and Mrázek, '03	Arch. mikr. Anat., 62, p. 431
Lumbricus agricola.		16 ♂ (= 64 el)	16 ♂ (= 32 el)	16 ♂		Bugnion and Popoff, '05	Arch. Zool. exper. et gen., Ser. IV, vol. 2, p. 339
Lumbricus terrestris......	32 spg	16 ♂	16 ♂	16 ♂		Calkins, '95	Jour. Morph., 11, p. 271
Lumbricus (sp. not given)	32 spg	16 ♂	16 ♂			Meek, '13	Phil. Trans. Roy. Soc. London, 203B, p. 1
Mesenchytraeus flavus...		16 ♀				Vejdovský, '07	Kgl. böhm. Ges. Wiss. Prag, '07, p. 1

Mesenchytraeus setosus..	32 oog	16 ♀				Vejdovský, '07	Kgl. bohm. Ges. Wiss. Prag, '07, p. 1
Rhynchelmis limosella	64 cl	32 ♀	32 ♀	32 ♀		Vejdovský and Mrázek, '03 Vejdovský, '07	Arch. mikr. Anat. 62, p. 431 Kgl. bohm. Ges. Wiss. Prag, '07, p. 1
Tubifex rivulorum.......	ca 100 cl	ca 110 ♀				Gathy, '00	La Cellule, 17, p. 7
2. Polychaeta *a.* Archichaetopoda							
Saccocirrus major........	8 spg	4 ♂ 4 ♀	4 ♂ 4 ♀	4 ♀		Hempelmann, '13	Zoologica 26, p. 249 (=Festschrift für Chun)
Saccocirrus major........	18 spg 18 oog 18 cl	9 ♂ 9 ♀	9 ♂	9 ♂		Von Baehr, '13	Zool. Anz., 43, p. 10
Saccocirrus papillocercus.	6 cl					Pierantoni, '06	Mitt. Zool. St. Neapel, 18, p. 46
Saccocirrus papillocercus.	8 spg	4 ♂ 4 ♀	4 ♂ 4 ♀	4 ♀		Hempelmann, '13	Zoologica 26, p. 249 (= Festschrift für Chun)
b. Errantia							
Nereis limbata...........	20-30 cl	14 ♀				Bonnevie '07 Bonnevie, '08	Biol. Bull., 13, p. 57 Arch. Zellf., 2, p. 201
Platynereis megalops		14 ♀		14 ♀		Just, '15	Jour. Morph., 26, p. 217
Ophryotrocha puerilis	4 spg 4 oog 4 som 4 cl 8 in some blastulae	2 ♀	2 ♀	2 ♀		Korschelt, '95 Korschelt, '95	Verh. deutsch. Zool. Gesel., 5, p. 96 Zeits. wiss. Zool., 60, p. 543
Ophryotrocha puerilis....	8 spg 8 oog 8 som	4 ♂ 4 ♀	4 ♂			Schreiners, '06	Anat. Anz., 29, p. 465
Ophryotrocha puerilis....	8 spg 8 som	4 ♂	4 ♂			Gregoire and Deton, '06	La Cellule, 23, p. 433
Ophryotrocha puerilis....	4 (pairs) spg 4 (pairs) som	4 (pairs) ♂	2 (pairs) ♂	2 (pairs) ♂	2 to each pole in 1st	Dehorne, '10 Dehorne, '11	Zool. Anz., 36, p. 209 Arch. Zool. exper. et gen., Ser. V. vol. 9, p. 1

I. ANNELIDA—Continued

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Tomopteris elegans.......	10 oog	5♀				Senna, '11	Arch. ital. Anat. Emb., 9, p. 299
Tomopteris onisciformis..		4♀				W. Wallace, '04	Brit. Assoc. Adv. Sci., 73, p. 282
Tomopteris onisciformis..	18 spg	9♂				Schreiners, '06	Arch. Biol., 22, p. 1
			c. Sedentaria				
Amphitrite	22 cl 11 pa cl	11♀	11♀	11♀	2 p.b.s. and 11 pa cl. Sometimes no p.b. and 22 pa cl	J. W. Scott, '06	Jour. Exp. Zool., 3, p. 49
Aricia....................	18 cl 9 pa cl	9♀	9♀	9♀	2 p.b.s. and 9 pa cl. Sometimes 1 p.b. and 18 pa cl	Kostanecki, '09	Bull. Inter. Acad. Sci. Cracovie, '09, p. 238
Chaetopterus pergamentaceous................	18 cl	9♀	9♀	9♀		Mead, '98	Jour. Morph., 14, p. 181
Chaetopterus pergamentaceous................		9♀	9♀			Lillie, '06	Jour. Exp. Zool., 3, p. 153
Lanice conchylega .	6 (pairs) som	6 (pairs) ♂	3 (pairs) ♂	3♂		Dehorne, '11	Arch. Zool. exper. et. gen., Ser. V, vol. 9, p. 1
Sabellaria spinulosa	8 (pairs) spg 8 (pairs) som	8 (pairs) ♂ 8 (pairs) ♀	4 (pairs) ♂ 4 (pairs) ♀	4 (pairs) ♀		Dehorne, '10 Dehorne, '11	C. R. Acad. Sci. Paris, 150, pp. 1195 and 1625 Arch. Zool. exper. et gen., Ser. V, vol. 9, p. 1
Serpula crater...........		ca 14♀		ca 7♂ pron ca 7♀ pron		Soulier, '06	Arch. Zool. exper. et gen., Ser. IV, vol. 5, p. 403
			d. Appendix-Myzostomidae				
Myzostoma glabrum......	24 cl	12♀	12♀	12♂ pron 12♀ pron		Wheeler, '97	Arch. Biol., 15, p. 1

c. Gephyrea

1. Armata

Thalassema mellita	24 cl	12 ♀				Griffen, '99	Jour. Morph., 15, p. 583
Thalassema mellita	12 pa cl	12 ♀	12 ♀	12 ♀		Lefevre, '06 Lefevre, '07	Science, 23, p. 522 Jour. Exp. Zool., 4, p. 91
Thalassema mellita.......		12+ ♀	16–17 ♀			Bonnevie, '08	Arch. Zellf., 2, p. 201

2. Inermia

Phascolosoma gouldii... Phascolosoma vulgare..	20 cl (= 10 bivalent in vulgare)	10 ♀	10 ♀			Gerould, '04 Gerould, '06	Arch. Zool. exper. et gen. Ser. IV, vol. 2, p. XVII Zool. Jahrb., 23, p. 77
Sipunculus nudus........		10 ♀			Observation of Francotte	Gerould, '06	Zool. Jahrb., 23, p. 77

d Hirudinea

Clepsine complanata.....		20 ♀				Gathy, '00	La Cellule, 17, p. 7
Nephelis vulgaris = Herpobdella atomaria......	16 oog	8 ♀				Jörgensen, '08	Arch. Zellf., 2, p. 279
Piscicola		16 ♀				Jörgensen, '13	Arch. Zellf., 10, p. 127

II. ARTHROPODA

a. Arachnida

1. Acarida

Ixodes reduvius..........	ca 28 spg	14 ♂				Nordenskiöld, '09	Zool. Anz., 34, p. 511
Pediculopsis graminum...	4 cl (sometimes fused)			2 ♂ pron 2 ♀ pron	= karyomeres	Reuter, '09	Acta Soc. Sc. Fen., 37, no. 7, p. 1

2. Araneida

Agalena naevia...........	52? spg			25, 27? ♂	2 X to pole in 1st. From correction in '09	L. B. Wallace, '00 L. B. Wallace, '05 L. B. Wallace, '09	Anat. Anz., 18, p. 327 Biol. Bull., 8, p. 169 Biol. Bull., 17, p. 120
Agalena naevia...........		ca 15 ♂				Painter, '14	Zool. Jahrb., 38, p. 509
Amaurobius sylvestris....			17+ ♂		X to pole in 1st. Supernumeraries	Painter, '14	Zool. Jahrb., 38, p. 509
Anyphaena saltibunda...		10 ♂			X to pole in 1st	Painter, '14	Zool. Jahrb., 38, p. 509

II ARTHROPODA (Continued)

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Callipes imbecilla		11♂			X to pole in 1st	Painter, '14	Zool. Jahrb., 38, p. 509
Clubiona		22-26♂				Carnoy, '85	La Cellule, 1, p. 189
Dugesiella hentzi		22♂			X to pole in 1st	Painter, '14	Zool. Jahrb., 38, p. 509
Dolomedes fontanus		13♂			Double X to pole in 1st	Painter, '14	Zool. Jahrb., 38, p. 509
Epeira sclopetaria	23 spg	12♂	11, 12♂	11, 12♂	X to pole in 1st	Berry, '06	Biol. Bull., 11, p. 193
Lycosa communis		11♂			X to pole in 1st	Painter, '14	Zool. Jahrb., 38, p. 509
Lycosa insopita..........	28 spg	13♂	13♂ (12-15)		Pair small chroms. in spg usually absent in spc. Equal XY	Montgomery, '05	Proc. Acad. Nat. Sci. Phila., 57, p. 162
Maevia vittata	28+ spg 29+ oog	15♂			X to pole in 1st	Painter, '14	Zool. Jahrb., 38, p. 509
Oxyopes salticus		11♂			X to pole in 1st. Supernumeraries	Painter, '14	Zool. Jahrb., 38, p. 509
Tegenaria atrica		18-24♂				Carnoy, '85	La Cellule, 1, p. 189
Tegenaria sp?		6-12♂				Carnoy, '85	La Cellule, 1, p. 189
Theridium tepidariorum	21 el	12♀	12♀	12♀		Montgomery, '07	Zool. Jahrb., 25, p. 237
Xysticus triguttatus..		11♂			X to pole in 1st	Painter, '14	Zool. Jahrb., 38, p. 509

3. Scorpionidae

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Buthus eupeus	ca 22 spg					Sokolow, '13	Arch. Zellf., 9, p. 399
Euscorpius carpathicus.	70-81 spg	28-40♂ (probably 32)	28-40♂			Sokolow, '13	Arch. Zellf., 9, p. 399
Scorpio occitanus (= Buthus)		22-28♂				Carnoy, '85	La Cellule 1, p. 189

4. Appendix—Tardigrada

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Macrobiotus lacustris	10 el	5♀	5♀	5♀		Von Wenck, '14	Zool. Jahrb. Abt. f. Anat., 37, p. 465

b. CRUSTACEA

1. *Entomostraca*

a. Cirrepedia

Lepas anatifera			4–12♀ (mostly 4 or 5)			Groom, '94	Phil. Trans. Roy. Soc., 185 B, p. 119

b. Copepoda

Anomalocera patersonii	16 (double) oog 32 som	16♀ (= 64 el)	16♀ (= 32 el)	16♀		Vom Rath, '95	Arch. mikr. Anat., 46, p. 168
Calanus gracilis		16♀				Konrhauser, '15	Arch. Zellf., 13, p. 399
Canthocamptus staphylinus	24 oog ca 12 (double) el	12♀ (= 48 el)	6 pairs ♀ (= 24 el)	6♀ (= 12 el)		Häcker, '92 Häcker, '95	Zool. Jahrb., 5, p.211 Arch. mikr. Anat., 45, p. 200
Canthocamptus staphylinus		12♀	12♀	12♀	Ditetrads	Matscheck, '09 Matscheck, '10	Zool. Anz., 34, p. 42 Arch. Zellf., 5, p. 36
Canthocamptus staphylinus		12♀ (double)				Krüger, '11	Arch. Zellf., 6, p. 165
Canthocamptus trispinosus		11♀ (double)				Krüger, '11	Arch. Zellf., 6, p. 165
Cyclops affinis		7♀			6 ditetrads + 1 heterochromosome	Matscheck, '10	Arch. Zellf., 5, p. 36
Cyclops albidus	14 som		7♀		Ditetrads	Braun, '09	Arch. Zellf., 3 p. 449
Cyclops albidus		7♀			Ditetrads	Matscheck, '09 Matscheck, '10	Zool. Anz., 34, p. 42 Arch. Zellf., 5, p. 36
Cyclops albidus	14					Chambers, '12	Univ. Toronto Studies, 14, p. 1
Cyclops bicuspidatus	18 som	9♀			Ditetrads	Braun, '09	Arch. Zellf., 3, p. 449
Cyclops bicuspidatus		9♀			Ditetrads	Matscheck, '09 Matscheck, '10	Zool. Anz., 34, p. 42 Arch. Zellf., 5, p. 36
Cyclops bicuspidatus	18					Chambers, '12	Univ. Toronto Studies 14, p. 1.
Cyclops bicuspidatus var. odessana	18 som	9♀			Ditetrads	Braun, '09	Arch. Zellf., 3, p. 449
Cyclops bicuspidatus var. odessana		9♀			Ditetrads	Matscheck, '10	Arch. Zellf., 5, p. 36

II. ARTHROPODA—Continued

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Cyclops brevicornis see Cyclops viridis							
Cyclops diaphanus.......	12 som	6♀			Ditetrads	Braun, '09	Arch. Zellf., 3, p. 449
Cyclops distinctus see fuscus distinctus							
Cyclops dybowskii.......	18 som	9♀			Ditetrads	Braun, '09	Arch. Zellf., 3, p. 449
Cyclops dybowskii		9♀			Ditetrads	Matscheck, '09 Matscheck, '10	Zool. Anz., 34, p. 42 Arch. Zellf., 5, p. 36
Cyclops fuscus...........	14 som	7♀			Ditetrads	Braun, '09	Arch. Zellf., 3, p. 449
Cyclops fuscus...........	7 oog	7♀			Ditetrads	Matscheck, '10	Arch. Zellf., 5, p. 36
Cyclops fuscus...........	14					Chambers, '12	Univ. Toronto Studies 14, p. 1
Cyclops fuscus var. distinctus (possibly hybrid fuscus x albidus)...	11 som	6♀			Ditetrads	Braun, '09	Arch. Zellf., 3, p. 449
Cyclops fuscus var. distinctus.................		6, 7♀			5 or 6 ditetrads + 1 heterochromosome	Matscheck, '10	Arch. Zellf., 5, p. 36
Cyclops fuscus var. distinctus.................	11 prim. germ cells 11 cl					Amma, '11	Arch. Zellf., 6, p. 497
Cyclops gracilis...........	6 som	3♀			Ditetrads	Braun, '09	Arch. Zellf., 3, p. 449
Cyclops gracilis...........	6 cl	3♀	3♀	3♂ pron 3♀	Ditetrads	Matscheck, '09 Matscheck, '10	Zool. Anz., 34, p. 42 Arch. Zellf., 5, p. 36
Cyclops insignis..........	22 som	11♀			Ditetrads	Braun, '09	Arch. Zellf., 3, p. 449
Cyclops insignis..........		11♀			Ditetrads	Matscheck, '10	Arch. Zellf., 5, p. 36
Cyclops insignis..........	22 prim. germ cells					Amma, '11	Arch. Zellf., 6, p. 497
Cyclops leuckarti.........	14 som	7♀			Ditetrads	Braun, '09	Arch. Zellf., 3, p. 449

Cyclops leuckarti		7♀			Ditetrads	Matscheck, '10	Arch. Zellf., 5, p. 36
Cyclops modestus	8					Chambers, '12	Univ. Toronto Studies 14, p. 1
Cyclops phaleratus	13 som	7♀			6 ditetrads + 1 heterochromosome	Braun, '09	Arch. Zellf., 3, p. 449
Cyclops phaleratus	7 Keimpolster	7♀	6, 7♀	6, 7♀	6 ditetrads + 1 heterochrom. Latter to pole in 1st	Matscheck, '09 Matscheck, '10	Zool. Anz., 34, p. 42 Arch. Zellf., 5, p. 36
Cyclops prasinus	11 som	6♀			5 ditetrads + 1 heterochromosome	Braun, '09	Arch. Zellf., 3, p. 449
Cyclops prasinus		6♀			5 ditetrads + 1 heterochromosome	Matscheck, '10	Arch. Zellf., 5, p. 36
Cyclops serrulatus	14 som	7♀		6, 7♀	6 ditetrads + 1 m-chrom. Latter to either pole in 2nd	Braun, '09	Arch. Zellf., 3, p. 449
Cyclops serrulatus		8♀			6 ditetrads + 2 heterochroms	Matscheck, '10	Arch. Zellf., 5, p. 36
Cyclops signatus	8 oog 8 cl (double)	8♀ (= 16 el)	4♀ (= 8 el)	4♂ 4♀		Häcker, '90 Häcker, '92	Zool. Anz., 13, p. 551 Zool. Jahrb., 5, p. 211
Cyclops strenuus	8 oog 8 cl (double)	8♀ (= 16 el)	4♀ (= 8 el)	4♂ 4♀		Häcker, '90 Häcker, '92 Häcker, '93 Häcker, '94	Zool. Anz., 13, p. 551 Zool. Jahrb., 5, p. 211 Arch. mikr. Anat., 41, p. 452 Arch. mikr. Anat., 43, p. 759
Cyclops strenuus	22–24 cl	11–12♀		11–12♂ pron 11–12♀ pron		Rückert, '94 Rückert, '95	Anat. Hefte, 4, p. 261 Arch. mikr. Anat., 45 p. 339
Cyclops strenuus	ca 20 spg	11♀	11♀			Lerat, '05	La Cellule, 22, p. 161
Cyclops strenuus	22 som	11♀ 14♀			Ditetrads. May be 2 varieties	Braun, '09	Arch. Zellf., 3, p. 449
Cyclops strenuus		11♀			Ditetrads	Matscheck, '10	Arch. Zellf., 5, p. 36
Cyclops strenuus	22 cl					Amma, '11	Arch. Zellf., 6, p. 497
Cyclops vernalis	10 som	5♀			Ditetrads	Braun, '09	Arch. Zellf., 3, p. 449
Cyclops vernalis		5, 6♀			5 ditetrads or same + 1 heterochrom	Matscheck, '09 Matscheck, '10	Zool. Anz., 34, p. 42 Arch. Zellf., 5, p. 36

II. ARTHROPODA—Continued

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Cyclops viridis = brevicornis..................	12 cl (double) = 24	12♀ (= 24)	6 pairs ♀ (= 12)	6♂ pron (= 12) 6♀		Häcker, '95	Arch. mikr. Anat., 46, p. 579
						Häcker '97	Biol. Centralb., 17, p. 721
						Häcker, '03	Jen. Zeits., 30, p. 297
						Häcker, '04	Zool. Jahrb., 7, suppl., p. 161
Cyclops viridis	12 cl 14 cl				2 varieties	Schiller, '08	Zool. Anz., 32, p. 616
Cyclops viridis	12 som	6♀			Ditetrads	Braun, '09	Arch. Zellf., 3, p. 449
Cyclops viridis	12 spg 12 oog					Krimmel, '10	Zool. Anz., 35, p. 778
Cyclops viridis		6♀			Ditetrads	Matscheck, '10	Arch. Zellf., 5, p. 36
Cyclops viridis	12 cl	6♀			Biserial	Amma, '11	Arch. Zellf., 6, p. 497
Cyclops viridis . . .	12					Chambers, '12	Univ. Toronto Studies 14, p. 1
Cyclops viridis var. americanus............	10 spg 10 oog 10 som	5♂ 5♀	5♂ 5♀	5♂ 5♀	Biserial	Chambers, '12	Univ. Toronto Studies, 14, p. 1
Cyclops viridis var. brevispinosus.............	4 oog	2♀	2♀		Biserial	Chambers, '12	Univ. Toronto Studies, 14, p. 1
Cyclops viridis var. parcus.....................	6 oog	3♀	3♀	3♂ pron 3♀	Biserial	Chambers, '12	Univ. Toronto Studies, 14, p. 1
Diaptomus castor........		15♀	17♀		1 heterochrom = 3 elements, are separate in 2nd and divide in both divisions	Häcker and Matscheck, '08 Matscheck, '09 Matscheck, '10	Verh. deutsch. Zool. Ges., 18, p. 110 Zool. Anz., 34, p. 42 Arch. Zellf., 5, p. 36

Diaptomus castor	34 cl			17♂ pron 17♀ pron		Amma, '11	Arch. Zellf., 6, p. 497
Diaptomus coeruleus.....		14♀				Häcker and Matscheck, '08 Matscheck, '09	Verh. deutsch. Zool. Ges., 18, p. 110 Zool. Anz., 34, p. 42
Diaptomus coeruleus	28 spg (14-28) 28 oog 28 som (14-28)					Krimmel, '10	Zool. Anz., 35, p. 778
Diaptomus coeruleus.....	28 cl			14♂ pron 14♀ pron		Amma, '11	Arch. Zellf., 6, p. 497
Diaptomus denticornis...	32 cl					Häcker, '03	Jen. Zeits., 30, p. 297
Diaptomus denticornis		17♀			Ditetrads	Matscheck, '09 Matscheck, '10	Zool. Anz., 34, p. 42 Arch. Zellf., 5, p. 36
Diaptomus gracilis	32 cl	16♀				Rückert, '94	Anat. Hefte 4, p. 261
Diaptomus gracilis		17♀			Ditetrads	Häcker and Matscheck, '08 Matscheck, '09	Verh. deutsch. Zool. Ges., 18, p. 110 Zool. Anz., 34, p. 42
Diaptomus laciniatus .		16♀			Ditetrads	Matscheck, '09 Matscheck, '10	Zool. Anz., 34, p. 42 Arch. Zellf., 5, p. 36
Diaptomus salinus		17♀			Ditetrads	Matscheck, '09 Matscheck, '10	Zool. Anz., 34, p. 42 Arch. Zellf., 5, p. 36
Diaptomus sp? (Japanese)....................	8 spg 8 oog 8 cl	8♂ 8♀	8♂ 8♀	4♂ 4♀	4 chroms to each pole in 2nd	Ishikawa, '91	Coll. Sci. Imp. Univ. Japan, 5, p. 1
Euchaeta marina		12♀ (= 48 el)	12♀ (= 24 el)	12♀		Vom Rath, '95	Arch. mikr. Anat., 46, p. 168
Euchaeta marina		17♀				Kornhauser, '15	Arch. Zellf., 13, p. 309
Hersilia apodiformis	24 spg 24 oog 24 cl	12♂ 12♀	12♂		Sometimes 13 in 1st; one pair fails to conjugate	Kornhauser '15	Arch. Zellf., 13, p. 309
Heterocope robusta		16♀				Rückert, '94	Anat. Hefte, 4, p. 261
Heterocope saliens		16♀				Matscheck, '09 Matscheck, '10	Zool. Anz., 34, p. 42 Arch. Zellf., 5, p. 36
Heterocope weismanni		16♀				Häcker and Matscheck, '08 Matscheck, '09 Matscheck, '10	Verh. deutsch. Zool. Ges., 18, p. 110 Zool. Anz., 34, p. 42 Arch. Zellf., 5, p. 36

II. ARTHROPODA—Continued

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Laemargus muricatus.....	16 spg 16? oog	8♂	8♂	8♂		McClendon, '06	Biol. Bull., 12, p. 37
Lichomolgus forficula		10♀				Kornhauser, '15	Arch. Zellf., 13, p. 399
Mytilicola intestinalis.....	8 spg 8 oog	16♂	8♂	4♂		Steuer, '03	Arb. Zool. Inst. Wien, 15, p. 1
Orthagoriscicola muricata	16 oog	8♀				McClendon, '06 McClendon, '10	Biol. Bull., 12, p. 37 Arch. Zellf., 5, p. 229
Nitocra hibernica.........		8♀ (double)				Krüger, '11	Arch. Zellf., 6, p. 165
Pandarus sinuatus........	16 spg 16 oog	8♂ 8♀	8♀ 8♀	8♂		McClendon, '06	Biol. Bull., 12, pp. 37 and 53
						McClendon, '07 McClendon, '10	Biol. Bull., 13, p. 114 Arch. Zellf., 5, p. 229
Paracalanus parvus		6♀				Moroff, '09	Arch. Zellf., 2, p. 432
Sapphirina sp?..	16 spg					Kornhauser, '15	Arch. Zellf., 13, p. 399
c. Ostracoda							
Cypris fuscata... . .	24 cl	24♀			Pa egg; 1 p.b., no reduction	Schleip '09	Arch. Zellf., 2, p. 390
Cypris incongruens.......	12 pa cl	12♀			Pa; 1 p.b.	Woltereck, '98	Zeit. wiss. Zool., 64, p 596
Cypris incongruens.......	12 pa cl	12♀			Pa; 1 p.b., no reduction. No X	Müller-Calé, '13	Zool.-Jahrb., 36, p. 113.
Cypris reptans	12 pa cl	12♀			Pa; 1 p.b.	Woltereck, '98	Zeit. wiss. Zool., 64, p. 596
Notodromas monacha = Cyprois monacha.......	13–16 oog 16 cl	8♀	8♀	8♂ 8♀		Schleip, '09	Arch. Zellf., 2, p. 390
Notodromas monacha....	16 cl	8♂	8♂	8♂		Schmalz, '11 Schmalz, '12	Zool. Anz., 37, p. 462 Arch. Zellf., 8, p. 407

d. Phyllopoda

1. Cladocera

Bythotrephes longimanus	4? cl			2? ♀		Weismann and Ishikawa, '89	Zool. Jahrb., 4, p. 155
Daphnia pulex........	8 spg 8 som 8 cl	8 ♀			Pa egg; 1 p.b., no reduction	Kühn, '08	Arch. Zellf., 1, p. 538
Daphnia pulex	8-10 spg 8-10 som	4-5 ♂	4-5 ♂			Taylor, '14	Zool. Anz., 45, p. 21
Moina paradoxa Moina rectirostris		4 ♀	4 ♀		Winter egg	Weismann and Ishikawa, '89	Zool. Jahrb., 4, p. 155
Polyphemus pediculus .	8 spg 8 som 8 cl	8 ♀				Kühn, '08	Arch. Zellf., 1, p. 538
Simocephalus vetulus		8 ♂	ca 8 ♂		No X	Chambers, '13	Biol. Bull., 25, p. 134

2. Euphyllopoda

Apus	1 oog				Monomeric division	Moore, '93	Q. J. M. S., 35, p. 259
Artemia salina ...		30-40 ♀			Pa; 1 p.b.	Weismann and Ishikawa, '88	Zool. Jahrb., 3, p. 575
Artemia salina	84 cl 168 cl	84 ♀	84 ♀	84 ♀	Pa; pronucleus may be fertilized by 2nd p.b. (= 168)	Brauer, '93 Brauer, '94	Zool. Anz., 16, p. 138 Arch. mikr. Anat., 43, p. 162
Artemia salina		84 ♀		84 ♀	Pa; 1 p.b.	Petrunkewitsch, '02	Anat. Anz., 21, p. 256
Artemia salina	80-90 oog (= 84?) 70-90 som	84 ♀				Fries, '09	Arch. Zellf., 4, p. 44
Artemia salina var. bivalens parthenogenetica di Capo d'Istria....	84 cl	84 ♀			Pa; 1 p.b.	Artom, '08	Biologica 1, pp. 5 and 495
						Artom, '11	Arch. Zellf., 7, p. 277
						Artom, '11	Biol. Centralb., 31, p. 104
						Artom, '12	Arch. Zellf., 9, p. 87
Artemia salina var. univalens sessuata di Cagliari..............	42 cl	21 ♀	21 ♀		Sexual	Artom, '08	Biologica 1, pp. 5 and 495
						Artom, '11	Arch. Zellf., 7, p. 277
						Artom, '11	Biol. Centralb., 31, p. 104
						Artom, '12	Arch. Zellf., 9, p. 87

II. ARTHROPODA—Continued

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Branchipus		10♂ (double)	10♂ (double)			Moore, '93	Q. J. M. S., 35, p. 259
Branchipus grubii	24 oog 24 cl	12♀	12♀	12♀		Brauer, '92	Abh. Akad. Wiss. Berlin, Physik. Abt., '92, p. 1
						Brauer, '94	Arch. mikr. Anat., 43, p. 162
Branchipus grubii	22–24 oog 24 som	12♀		12♂ pron 12♀ pron		Fries, '09	Arch. Zellf., 4, p. 44
2. *Malacostraca* a. Arthrostraca 1. Amphipoda							
Talorchestia longicornis		18♂				Nichols, '09	Jour. Morph., 20, p. 461
2. Isopoda							
Asellus aquaticus	20–30 spg	8–10♂				Carnoy, '85	La Cellule, 1, p. 189
Idotea irrorata		28♂				Nichols, '09	Jour. Morph., 20, p. 461
Oniscus asellus		16♂				Nichols, '02	Proc. Amer. Phil. Soc., 41, p. 77
						Nichols, '09	Jour. Morph., 20, p. 461
b. Decapoda 1. Brachyura							
Carcinus menas		30–40♂				Carnoy, '85	La Cellule, 1, p. 189
Menippe mercenaria	51–80 spg		25–28♀			Binford, '13	Jour. Morph., 24, p. 147
2. Macrura							
Astacus fluviatilis		ca 58♂				Prowazek, '02	Zeits. wiss. Zool., 71, p. 445
Cambarus immunis?		104♂	104♂		A clump of 8 chroms	Fasten, '14	Jour. Morph., 25, p. 587

Cambarus virilis.........	200 spg	100♂	100♂			Fasten, '14	Jour. Morph., 25, p. 587
Crangon cataphractus....		40–44♂				Carnoy, '85	La Cellule, 1, p. 189
Eupagurus prideauxii.....		12♀	12♀			Weismann and Ishikawa, '88	Zool. Jahrb., 3, p. 575
Euphasidae..............	38 cl					Taube, '09	Zeits. wiss. Zool., 92, p. 427
Hippa talpoides..........		60♂				Nichols, '09	Jour. Morph., 20, p. 461
Homarus.................		18♂				Labbé, '04	C. R. Acad. Sci. Paris, 138, p. 96
c. Stomatopoda							
Squilla mantis............		20–24♂				Carnoy, '85	La Cellule, 1, p. 189
c. INSECTA *1. Aptera* a. Collembola							
Anurida maritima........	8 oog 8? cl				Unfavorable for maturation	Claypole, '98	Jour. Morph., 14, p. 219
Orchesella villosa.........		6?♀				Lécaillon, '01	Arch. d'Anat. mikr., 4, p. 471
Podura aquatica..........	8 oog					Willem, '00	Mém. couronées Acad. roy. Belgique 58, no. 3, p. 1
2. Coleoptera a. Buprestidae							
Euchroma gigantea.......		13♂			XY to poles in 1st	Nichols, '10	Biol. Bull., 19, p. 167
Spruce-borer (unidentified)...................		10♂	10♂		XY to poles in 1st	Stevens, '06	Carnegie Inst. Pub., 36 II, p. 33
Spruce-borer (another sp).....................		11♂				Stevens,' 06	Carnegie Inst. Pub., 36 II, p. 33

II. ARTHROPODA—Continued

b. Carabidae

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Anomoglossus emarginatus		19♂	18, 19♂	18, 19♂	X to pole in 1st	Stevens, '06	Carnegie Inst. Pub., 36, II, p. 33
Chlaenius aestivus		17♂			XY to poles in 1st	Stevens, '06	Carnegie Inst. Pub., 36, II, p. 33
Chlaenius pennsylvanicus		10♂			XY to poles in 1st	Stevens, '06	Carnegie Inst. Pub., 36, II, p. 33
Galerita bicolor	30 spg				XY	Stevens, '06	Carnegie Inst. Pub., 36, II, p. 33
c. Cerambycidae							
Cylene robinia	20 spg	10♂			XY to poles in 1st	Stevens, '09	Jour. Exp. Zool., 6, p. 101
Tetraopes tetraophthalmus	20 spg	10♂			XY to poles in 1st	Stevens, '09	Jour. Exp. Zool., 6, p. 101
d. Chrysomelidae							
Adimonia tanaceti		12 (at least) ♀				Henking, '92	Zeit. wiss. Zool., 54, p. 1
Agelastica alni	24–25 spg 24–30 cl	ca. 12♂ (= 16 -17 el) 12♀	ca. 12♂ (= 16 -17 el) 12♀			Henking, '92	Zeit. wiss. Zool., 54, p. 1
Blepharida rhois		16♂	16♂	16♂	XY to poles in 1st	Stevens, '06	Carnegie Inst. Pub., 36, II, p. 33
Chelymorpha argus	22 spg 22♀ som	11♂	11♂		XY to poles in 1st XX in ♀ diploid	Stevens, '06	Carnegie Inst. Pub. 36, II. p. 33

e. Cincindellidae							
Cincindella primeriana..	20 spg	10♂	10♂		XY (tripartite) to poles in 1st	Stevens, '06	Carnegie Inst. Pub., 36, II, p. 33
Cincindella vulgaris......	22 spg				Trilobed XY in 1st	Stevens, '09	Jour. Exp. Zool., 6, p. 101
f. Coccinellidae							
Adalia bipunctata........	20 spg	10♂	10♂		XY to poles in 1st	Stevens, '06	Carnegie, Inst. Pub., 36, II, p. 33
Epilachna borealis........	18 spg	9♂	9♂		XY to poles in 1st	Stevens, '06	Carnegie Inst. Pub., 36, II, p. 33
Coccinellidae, (a number of sp)..............	20 spg	10♂	10♂		XY to poles in 1st	Stevens, 09	Jour. Exp. Zool., 6, p. 101
g. Dytiscidae							
Colymbetes fuscus........	35–37 oog					Gunthert, '10	Zool. Jahrb., 30, p. 301
Cybister roeselii..........	ca. 22 spg	12?♂(11–15)	12♂	6♂		Voinov, '03	Arch. Zool. exp. et gen. ser. IV, vol. 1, p. 173
Dytiscus................	ca 40 oog ca. 40♀ som					Giardina, '01	Intern. Monats. Anat. u. Phys., 18, p. 417
Dytiscus circumcinctus...	38 spg	19♂	19♂	19♂		Schäfer, '07	Zool. Jahrb., 23, p. 535
Dytiscus marginalis......	ca. 40 spg (36–41)				No X	Henderson, '07	Zeit. wiss. Zool., 87, p. 644
Dytiscus marginalis	38 spg	19♂	19♂	19♂	X divides in both divisions. 2X in spg.	Schäfer, '07	Zool. Jahrb., 23, p. 535
Dytiscus marginalis......	40 oog					Debaisieux, '09	La Cellule, 25, p. 207
h. Elateridae							
Elater sp? I.............	19 spg 20 oog	10♂			X to pole in 1st	Stevens, '06	Carnegie Inst., Pub., 36, II, p. 33
Elater sp? II............	19 spg				X	Stevens, '06	Carnegie Inst. Pub., 36, II, p. 33
Limoneus griseus.........	17 spg	9♂	8, 9♂	8, 9♂	X to pole in 1st	Stevens, '09	Jour. Exp. Zool., 6, p. 101

II. ARTHROPODA—Continued

Hydrophilidae

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	TD	REMARKS	OBSERVER	REFERENCE
Hydrophilus piceus.......		10–12♂	5–6♂			Carnoy, '85	La Cellule 1, p. 189
Hydrophilus piceus.......	16 spg	16♂ (= 32 el)	8♂ (= 16 el)	[illegible]	Tetrads and dyads = separate elements	Vom Rath, '92	Arch. mikr. Anat., 40, p. 102
Hydrophilus piceus.......	30 som	15♂(gemini)	15♂			Arnold, '08	Arch. Zellf., 2, p. 181
Chrysochus auratus		13♂			XY to poles in 1st	Stevens, '09	Jour. Exp. Zool., 6, p. 101
Chrysomela similis.......		12♂	11, 12♂	11, 12♂	X to pole in 1st	Stevens, '09	Jour. Exp. Zool., 6, p. 101
Coptocycla aurichalcea...	22 spg	11♂	11♂	11♂	XY to poles in 1st	Nowlin, '06	Jour. Exp. Zool., 3, p. 583
Coptocycla clavata.....	18 spg				XY	Stevens, '09	Jour. Exp. Zool., 6, p. 101
Coptocycla guttata.......	18 spg	9♂	9♂	9♂	XY to poles in 1st	Nowlin, '06	Jour. Exp. Zool., 3, p. 583
Crioceris asparagi.........		8♀	8♀			Henking, '92	Zeit. wiss. Zool., 54, p. 1
Diabrotica 12-punctata. Diabrotica soror........	19 spg	10♂	9, 10♂	9, 10♂	X to pole in 1st		
	20 spg	11♂	9, 10, 11♂	9, 10, 11♂	X; S to pole in 1st or 2nd		
	21 spg	12♂	9–12♂	9–12♂	X; 2S		
		13♂	9–13♂		X; 3S		
			9–14♂		X; 4S	Stevens, '08	Jour. Exp. Zool., 5, p. 453
Diabrotica vittata........	21 spg	11♂	10, 11♂	10, 11♂	X to pole in 1st	Stevens, '08	Jour. Exp. Zool., 5, p. 453
Diabrotica vittata	21, 22 som					Hoy, '14	Biol. Bull., 27, p. 45
Donacia (sericea?)			15♂			Henking, '92	Zeit. wiss. Zool., 54, p. 1
Doryphora clivicolis......		17♂	17♂		XY to poles in 1st	Stevens, '09	Jour. Exp. Zool., 6, p. 101
Doryphora decemlineata.	36 spg	18♂	18♂			Stevens, '06	Carnegie Inst. Pub. 36, II, p. 33

Haltica chalybea.........	22 spg	11♂			XY to poles in 1st	Stevens, '09	Jour. Exp. Zool., 6, p. 101
Lema trilineata	32 spg		16♂		XY to poles in 1st	Stevens, '09	Jour. Exp. Zool., 6, p. 101
Leptinotarsa signaticolis..		17♂	16, 17♂	16, 18♂	Bivalent X to pole in 1st. Parts separate in 2nd anaph.	Wieman, '10	Jour. Morph., 21, p. 135
Lina laponica............		17♂			XY to poles in 1st	Stevens, '09	Jour. Exp. Zool., 6, p. 101
Odontota dorsalis.........	16 spg 16♂ som	8♂	8♂		XY to poles in 1st. One testis, Y = 2 elements, 17 spg., 8, 9 2nd cyte	Stevens, '06	Carnegie Inst. Pub., 36, II, p. 33
Trirhabda canadense.....	30 spg 30♀ som	15♂	15♂		XY to poles in 1st XX in ♀ diploid	Stevens, '06	Carnegie Inst. Pub., 36, II, p. 33
Trirhabda virgata........	28 spg, 28♂ som 28♀ som	14♂	14♂		XY to poles in 1st XX in ♀ diploid	Stevens, '06	Carnegie Inst. Pub., 36, II, p. 33
			j. Lampyridae				
Ellychnia corrusca........	19 spg				X	Stevens, '06 Stevens, '09	Carnegie Inst. Pub., 36, II, p. 33 Jour. Exp. Zool., 6, p. 101
Lampyrus splendidula....		6–8♀	6–8♀			Henking, '92	Zeit. wiss. Zool. 54, p. 1
Photinus consanguineus Photinus pennsylvanicus..................	19 spg 20 oog 19♂ som	10♂	10♂	9, 10♂	X to pole in 2nd	Stevens, '09	Jour. Exp. Zool., 6, p. 101
			k. Melandryidae				
Penthe obliquata.........	16 spg		8♂		XY to poles in 1st	Stevens, '09	Jour. Exp. Zool., 6, p. 101
			l Meloidea				
Epicauta cinerea.. Epicauta pennsylvanica........	20 spg	10♂	10♂		XY to poles in 1st	Stevens, '09	Jour Exp Zool., 6, p 101

II. ARTHROPODA—Continued

m. Scarabaeidae

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Euphoria inda	20 spg	10♂	10♂	10♂	XY to poles in 1st	Stevens, '06	Carnegie Inst. Pub., 36, II, p. 33
Oryctes nasicornis........	12 spg	6♂	6♂	6♂		Prowazek, '02	Arb. Zool. Inst. Wien, 13, p. 223
			n. Silphidae				
Necrophorus sayi.........	13 spg	7♂	6, 7♂	6, 7♂	X to pole in 1st	Stevens, '09	Jour. Exp. Zool., 6, p. 101
Silpha americana.........	40 spg	20♂	20♂		XY to poles in 1st	Stevens. '06	Carnegie Inst. Pub., 36 II, p. 33
Silpha carinata...........	32 spg	16♂ (= 64 cl)	16♂(= 32 cl)	16♂		Holmgren, '02	Anat. Anz., 22, p. 194
			o. Staphylinidae				
Listotrophus cingulatus..	26 spg	13♂	13♂		XY to poles in 1st	Stevens, '09	Jour. Exp. Zool., 6, p. 101
Rove-beetle, brown.......	28 spg	14♂	14♂			Stevens, '09	Jour. Exp. Zool., 6, p. 101
Staphylinus violaceus		22♂	22♂		XY to poles in 1st	Stevens, '09	Jour. Exp. Zool., 6, p. 101
			p. Tenebrionidae				
Blaps lusitanica..........	35 spg (33 in some)	17♂ (16 in some)	17, 17 + X♂ (16 in some)		X to pole in 1st. X attached to another pair in 1st and 2nd	Fernández-Nonidez, '14	Trab. Mus. Nac. de C. Nat. de Madrid, Ser., Zool., No. 18, p. 1
Tenebrio molitor	20 spg 20 oog 20♂ spg 20♀ som	10♂	10♂	10♂	XY to poles in 1st XY in spg XX in oog	Stevens, '05	Carnegie Inst. Pub, 36, p. 1

3. *Diptera*

a. Anthomyiidae

Phorbia brassica..........		6♂			XY to poles in 1st	Stevens, '08	Jour. Exp. Zool., 5, p. 359

b. Cecidomyiidae

Miastor americana........		20–24♀			Paedogenetic. One polar body	Hegner, '14	Jour. Morph., 25, p. 375
Miastor metraloas........	20–24 oog 20–24 early cl 10–11 late cl	20–24♀			Paedogenetic. One polar body	Kahle, '08	Zoologica, 21, p. 1

c. Chironomidae

Chironomus confinis.... Chironomus riparius...		4–8♀				Hasper, '11	Zool. Jahrb., 31, p. 543

d. Culicidae

Anopheles punctipennis .	6 spg 6 oog	3♂	3♂	3♂	XY in ♂ attached to another pair. To poles in 1st. XX in ♀ attached to another pair.	Stevens, '11	Biol. Bull., 20, p. 109
Anopheles sp?............		6♂	6♂		XY to poles in 1st	Stevens, '11	Biol. Bull., 20, p. 109
Culex pipiens.............	6 spg 6 oog	3♂	3♂	3♂	No XY. (= pungens? '10)	Stevens, '10 Stevens, '11	Jour. Exp. Zool., 8, p. 207 Biol. Bull., 20, p. 109
Culex pipiens.............	6 spg (= 3 pairs)	3♂	3♂			Lomen, '14	Jen. Zeits., 45, p. 567
Culex pipiens.............	3 spg 3 and 6 oog 3 som	3♂	3♂	3♂		Taylor, '14	Q. J. M. S., 60, p. 377
Culex tarsalis.............	6 spg 6 oog	3♂	3♂	3♂	No XY	Stevens, '11	Biol. Bull., 20, p. 109
Theobaldia incidens	6 spg 6 oog	3♂	3♂		No XY	Stevens, '11	Biol. Bull., 20, p. 109

II. ARTHROPODA—Continued

e. Muscidae

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST CYTE	2ND CYTE	TID	REMARKS	OBSERVER	REFERENCE
Calliphora vomitoria	12 spg 12 oog	6♂	6♂	6♂	XY to poles in 1st	Stevens, '08	Jour. Exp. Zool., 5, p. 359
Lucilia caesar		6♂	6♂		XY to poles in 1st	Stevens, '08	Jour. Exp. Zool., 5, p. 359
Musca domestica	12 spg 12 oog	6♂	6♂	6♂	XY to poles in 1st	Stevens, '08	Jour. Exp. Zool., 5, p. 359
f. Muscidae acalyptratae							
Drosophila amoena	8 spg 8 oog		4♂		XY in ♂ diploid XX in ♀ diploid	Metz, '14	Jour. Exp. Zool., 17, p. 45
Drosophila ampelophila	8 spg 8 oog 8 cl	4♂	4♂	4♂	XY to poles in 1st X may be of two parts	Stevens, '08	Jour. Exp. Zool., 5, p. 359
Drosophila ampelophila	8 oog				XX in ♀ diploid	Metz, '14	Jour. Exp. Zool., 17, p. 45
Drosophila funebris	10 or 12 oog				Pair m-chroms, present (= 12) or absent (= 10)	Metz, '14	Jour. Exp. Zool., 17, p. 45
Drosophila quinaria	8 oog				XX in ♀ diploid	Metz, '14	Jour. Exp. Zool., 17, p. 45
Drosophila repleta	12 oog				XX in ♀ diploid	Metz, '14	Jour. Exp. Zool., 17, p. 45
Drosophila tripunctata	8 oog				XX in ♀ diploid	Metz, '14	Jour. Exp. Zool., 17, p. 45
Drosophila 2 sp?	8 oog				XX in ♀ diploid	Metz, '14	Jour. Exp. Zool., 17, p. 45
Drosophila 2 sp? (A and B)	12 oog				XX in ♀ diploid	Metz, '14	Jour. Exp. Zool., 17, p. 45
Drosophila sp? (C)	10 oog		5♂		XX in ♀ diploid	Metz, '14	Jour. Exp. Zool., 17, p. 45

Scatophaga pallida . . .	12 spg 12 oog	6♂	6♂	6♂	XY to poles in 1st	Stevens, '08	Jour. Exp. Zool., 5, p. 359
Tetanocera sparsa........	12 spg 12 oog	6♂	6♂	6♂	XY to poles in 1st	Stevens, '08	Jour. Exp. Zool., 5, p. 359
g. Sarcophagidae							
Sarcophaga sarraciniae....	12 spg 12♀ som	6♂	6♂	6♂	XY to poles in 1st	Stevens, '08	Jour. Exp. Zool., 5, p. 359
h. Syrphidae							
Eristalis tenax............	12 spg 12♂ som 12♀ som	6♂	6♂	6♂	XY to poles in 1st	Stevens, '08	Jour. Exp. Zool., 5, p. 359
4. Hemiptera a. Heteroptera 1. Belostomidae							
Zaitha sp? (fluminea or aurantiacum) . . .	24 spg	13♂	12♂	12♂	XY to poles in 2nd	Montgomery, '01 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Trans. Amer. Phil. Soc., 21, p. 97
2. Capsidae							
Calocoris rapidus	30 spg	16♂	15, 16♂		2X, one to pole in 1st, other in 2nd	Montgomery, '01 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Trans. Amer. Phil. Soc., 21, p. 97
Leptoterna dolabrata.....		17♂				Montgomery, '01	Trans. Amer. Phil. Soc., 20, p. 154
Lygus pratensis	prob. 35 spg	19♂	17, 18♂	17, 18♂	X to pole in 1st, XY to poles in 2nd. From correction in '06	Montgomery, '01 Montgomery, '06	Proc. Acad. Nat. Sci. Phila., 53, p. 261 Trans. Amer. Phil. Soc., 21, p. 97
Poecilocapsus goniphorus.		18♂	17♂	17♂	XY to poles in 2nd	Montgomery, '01 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Trans. Amer. Phil. Soc., 21, p. 97
Poecilocapsus lineatus....		18♂				Montgomery, '01	Trans. Amer. Phil. Soc., 20, p. 154

II. ARTHROPODA—Continued

3. Coreidae

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST CYTE	2ND CYTE	HD	REMARKS	OBSERVER	REFERENCE
Alydus eurinus	13 spg	7♂	7♂	6, 7♂	X to pole in 2nd	Montgomery, '01 Montgomery, '04 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Biol. Bull., 6, p. 137 Trans. Amer. Phil. Soc., 21, p. 97
Alydus pilosulus	13 spg	7♂	7♂	6, 7♂	X to pole in 2nd. From correction in '06	Montgomery, '01 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Trans. Amer. Phil. Soc., 21, p. 97
Alydus pilosulus	13 spg 14 oog	7♂	7♂	6, 7♂	X to pole in 2nd	Wilson, '05	Jour. Exp. Zool., 2, p. 507; 3, p. 1
Anasa armigera	21 spg	11♂			X to pole in 2nd From correction in '06	Montgomery, '01 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Trans. Amer. Phil. Soc., 21, p. 97
Anasa armigera	22 oog				XX in oog	Wilson, '09	Jour. Exp. Zool., 6, p. 69
Anasa tristis	22 spg	11♂	11♂	10, 11♂	Pair small chroms. in spg; to pole in 2nd and degenerate	Paulmier, '98 Paulmier, '99	Anat. Anz., 14, p. 514 Jour. Morph., 15 suppl. p. 223
Anasa tristis	21 spg 22 oog	11♂	11♂	10, 11♂	X to pole in 2nd. From correction in '06	Montgomery, '01 Montgomery, '04 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Biol. Bull., 6, p. 137 Trans. Amer. Phil. Soc., 21, p. 97
Anasa tristis	21 spg 22 oog	11♂	11♂	10, 11♂	X to pole in 2nd	Wilson, '05, '06 Wilson, '07 Wilson, '11	Jour. Exp. Zool., 2, pp. 371 and 507; 3, p. 1 Science, 25, no. 631, p. 191 Jour. Morph., 22, p. 71
Anasa tristis	22 spg	11♂	11♂	11♂	No accessory but nucleolus	Foot and Strobell, '07 Foot and Strobell, '07	Biol. Bull., 12, p. 119 Amer. Jour. Anat., 7, p. 279

Anasa tristis	21 spg			10, 11♂	X to pole in 2nd	Lefevre and McGill, '08	Amer. Jour. Anat., 7, p. 469
Anasa tristis.	21 spg, 21♂ cl 22 oog, 22♀ cl (One cl 23)	11♀	11♀		X in ♂ diploid XX in ♀ diploid	Morrill '10	Biol. Bull., 19, p. 79
Anasa tristis	21 spg 22 oog	11♂ (12 in two cases)	11♂		X to pole in 2nd	McClung and Pinney, '10	Kansas Univ. Sci. Bull., 5, p. 349
Anasa tristis	21, 22 som					Hoy, '14	Biol. Bull., 27, p. 45
Anasa sp? (from California)	21 spg 22 oog	11♂			X From correction in '06	Montgomery, '01 Montgomery, '04 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Biol. Bull., 6, p. 137 Trans. Amer. Phil. Soc., 21, p. 97
Archimerus alternatus (= calcarator?).........	15 spg 16 oog 15♂ cl 16♀ cl	8♂ 8♀	7, 8♂ 8♀	7, 8♂ 8♀	X to pole in 1st (♂) X in ♂ diploid. XX in ♀ diploid.	Morrill, '10	Biol. Bull., 19, p. 79
Archimerus calcarator	15 spg 16 oog	8♂	7, 8♂	7, 8♂	X to pole in 1st	Wilson, '05 Wilson, '09	Jour. Exp. Zool., 2, pp. 371 and 507 Jour. Exp. Zool., 6, p. 69
Catorintha............	25 spg				X	Wilson, '07	Science, 25, no. 631, p. 191
Chariesterus antennator		13♂	13♂	12, 13♂	X to pole in 2nd	Montgomery, '01 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Trans. Amer. Phil. Soc., 21, p. 97
Chariesterus antennator ?	21 spg				X to pole in 2nd. Species may be wrongly identified, labelled by Paulmier	Wilson, '05	Jour. Exp. Zool., 2, pp. 371 and 507
Chariesterus antennator.	25 spg 26 oog				X in spg. XX in oog	Wilson, '09	Jour. Exp. Zool., 6, p. 69
Chelinidea vittigera . . .	21 spg				X	Wilson, '07	Science, 25, no. 631, p. 191
Chelinidea vittigera ...	21 spg 22 oog 21♂ cl 22♀ cl (one spg 22)				X in ♂ diploid XX in ♀ diploid	Morrill, '10	Biol. Bull., 19, p. 79

II. ARTHROPODA—Continued

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST SP'CYTE	2ND SP'CYTE	SP'D	REMARKS	OBSERVER	REFERENCE
Corizus alternatus........	13 spg	7♂ (8 if 2 univalent)	7♂	6, 7♂	X to pole in 2nd. From correction in '06	Montgomery, '01	Proc. Acad. Nat. Sci. Phila., 53, p. 261
						Montgomery, '04	Biol. Bull., 6, p. 137
						Montgomery, '06	Trans. Amer. Phil. Soc., 21, p. 97
Corizus lateralis..........		7♂	7♂	6, 7♂	X to pole in 2nd	Montgomery, '01	Trans. Amer. Phil. Soc., 20, p. 154
						Montgomery, '06	Trans. Amer. Phil. Soc., 21, p. 97
Corynocoris distinctus....	25 spg 26 oog				X in spg. XX in oog	Wilson, '09	Jour. Exp. Zool., 6, p. 69
Euthoctha galeator.......	21 spg 22 oog				X in spg. XX in oog	Wilson, '07	Science, 25, no. 631, p. 191
						Wilson, '09	Jour. Exp. Zool., 6, p. 69
Harmostes reflexulus.....	13 spg	7♂ (8 if 2 univalent)	7♂	6, 7♂	X to pole in 2nd	Montgomery, '01	Trans. Amer. Phil. Soc., 20, p. 154
						Montgomery, '01	Proc. Acad. Sci. Phila., 53, p. 261
						Montgomery, '04	Biol. Bull., 6, p. 137
						Montgomery, '06	Trans. Amer. Phil. Soc., 21, p. 97
Harmostes reflexulus.....	13 spg 14 oog				X to pole in 2nd	Wilson, '06	Jour. Exp. Zool., 3, p. 1
Leptocoris trivittatus.....	13 spg 14 oog				X in spg. XX in oog	Wilson, '07	Science, 25, no. 631, p. 191
						Wilson, '09	Jour. Exp. Zool., 6, p. 69
Leptoglossus phyllopus...	21 spg 22 oog	11♂			X to pole in 2nd	Wilson, '07	Science, 25, no. 631, p. 191
						Wilson, '09	Jour. Exp. Zool., 6, p. 69
						Wilson, '11	Jour. Morph., 22, p. 71
Margus inconspicuus.....	23 spg 24 oog				X in spg. XX in oog	Wilson, '07	Science, 25, no. 631, p. 191
						Wilson, '09	Jour. Exp. Zool., 6, p. 69

Metapodius femoratus	22 spg	11♂ (1 triad)	11, 12♂	10, 11, 12♂	X to pole in 2nd. 2m to one pole, 1 m to other in 1st	Wilson, '07 Wilson, '09 Wilson, '10	Biol. Bull., 12, p. 303 Jour. Exp. Zool., 6, p. 147 Jour. Exp. Zool., 9, p. 53
	22 spg 22 oog	12♂	11♂	11♂	XY to poles in 2nd		
	23 spg 23 oog	13♂	11, 12♂	11, 12♂	XY and S to poles in 2nd. S may be attached to X or Y (= 11) or free (= 12) in 2nd metaphase		
	24 spg 24 oog	14♂	11, 12♂	11, 12, 13♂	XY and 2S (as above)		
	26 spg 26 oog	16♂	11-15♂	11-15♂	XY and 4S (as above)		
	28 oog				XY and 6S? (as above)		
Metapodiuos granulosus	22 spg 22 oog	12♂	11♂	11♂	XY to poles in 1st	Wilson, '07 Wilson, '09	Biol. Bull., 12, p. 303 Jour. Exp. Zool., 6, p. 147
	23 spg 23 oog	13♂	11, 12♂	11, 12♂	XY and S (as in M. femoratus)		
	24 spg 24 oog	14♂	11, 12♂	11, 12, 13♂	XY and 2S (as in M. femoratus)		
	25 spg 25 oog	15♂	11♂		XY and 3S (as in M. femoratus)		
	26 spg 26 oog	16♂	11-15♂	11-15♂	XY and 4S (as in M. femoratus)		
		17♂	11♂		XY and 5S (as in M. femoratus)		
Metapodius terminalis	21 spg	11♂	11♂	10, 11♂	X to pole in 2nd. From correction in '06	Montgomery, '01 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Trans. Amer. Phil. Soc., 21, p. 97
Metapodius terminalis.	21 spg	11♂	11♂	10, 11♂	X to pole in 2nd	Wilson, '07 Wilson, '09	Biol. Bull., 12, p. 303 Jour. Exp. Zool., 6, p. 147
	22 spg 22 oog	12♂	11♂	11♂	XY to poles in 2nd		
	23 spg 23 oog	13♂	11, 12♂	11, 12♂	XY and S (as in M. femoratus)		
	24 spg 24 oog	14♂	11, 12♂	11, 12, 13♂	XY and 2 S (as in M. femoratus)		

II. ARTHROPODA—Continued

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Metapodius terminalis	25 spg 25 oog	15♂	11♂		XY and 3S (as in M. femoratus)		
	26 spg 26 oog	16♂	11–15♂	11–15♂	XY and 4S (as in M. femoratus)		
Narnia	21 spg					Wilson, '07	Science, 25, no. 631, p. 191
Pachylis gigas	15 spg 16 oog	8♂			X in spg. XX in oog	Wilson, '07 Wilson, '09 Wilson, '11	Science, 25, no. 631, p. 191 Jour. Exp. Zool., 6, p. 69 Jour. Morph., 22, p. 71
Protenor belfragei	13 spg	7♂	7♂	6, 7♂	X to pole in 2nd	Montgomery, '01 Montgomery, '04 Montgomery, '06	Trans. Amer. Phil., Soc., 20, p. 154 Biol. Bull., 6, p. 137 Trans. Amer. Phil. Soc., 21, p. 97
Protenor belfragei	13 spg 14 oog	7♂	7♂	6, 7♂	X to pole in 2nd	Wilson, '05, '06 Wilson, '11	Jour. Exp. Zool., 2, p. 507; 3, p. 1 Jour. Morph., 22, p. 71
Protenor belfragei	13 spg 14 oog 13♂ cl 14♀ cl	7♀		7 pron.	X in ♂ diploid. XX in ♀ diploid	Morrill, '10	Biol. Bull., 19, p. 79
Syromastes marginatus	22 spg 22 ♀ som	11♂	10♂ (= 11, X a pole in metaphase)	10, 11♂	Bivalent X to pole in 2nd. Sperm with 10 not functional	Gross, '04 Gross, '04 Gross, '12	Verh. deutsch. Zool. Gesel., '04, p. 180 Zool. Jahrb., 20, p. 439 Zool. Jahrb., Abt. f. Zool., 32, p. 99
Syromastes marginatus	22 spg 24 oog	11♂	10♂ (= 12, X at pole in metaphase)	10, 12♂	Double X to pole in 2nd. X = 2 unequal parts, united in 1st metaphase	Wilson, '09 Wilson, '09	Jour. Exp. Zool., 6, p. 69 Biol. Bull., 16, p. 199
4. Corixidae							
Corixa verticalis		12♂				Montgomery, '01	Proc. Acad. Nat. Sci. Phila., 53, p. 261

5. Galgulidae

Galgulus oculatus (= Gelastocoris)...........	35 spg 38 oog 38♀ som	20♂	20♂	16, 19♂	XY to poles in 2nd X = 4 elements	Payne, '09	Biol. Bull., 16, p. 119

6. Hydrometridae

Hydrometra lacustris.....	11 spg	12♂	12♂		Large chrom of spg = 2 in spc.	Wilke, '07 Wilke, '13	Jen. Zeits., 35, p. 669 Arch. Zellf., 10, p. 203
Hydrometra paludum .	10 15 spg (prob. 12)	12♂ (few with 13 and 14)	12♂		X? divides in both divisions	Wilke, '12 Wilke' 13	Zool. Anz., 40, p. 216 Arch. Zellf., 10, p. 203
Hygotrechus sp?.........	21 spg	11♂	11♂	10, 11♂	X to pole in 2nd. From correction in '06	Montgomery, '01 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Trans. Amer. Phil. Soc., 21, p. 97
Limnotrechus marginatus		11♂	11♂		X to pole in 2nd	Montgomery, '01 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Trans. Amer. Phil. Soc., 21, p. 97

7. Lygaeidae

Cymus angustatus........			14♂		XY to poles in 2nd. From correction in '06	Montgomery, '01 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Trans. Amer. Phil. Soc., 21, p. 97
Cymus luridus...........		15♂				Montgomery, '01	Proc. Acad. Nat. Sci. Phila., 53, p. 261
Ichnodemus falicus.......	16 spg	9♂	8♂	8♂	XY to poles in 2nd. From correction in '06	Montgomery, '01 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Trans. Amer. Phil. Soc., 21, p. 97
Lygaeus bicrucis..........	14 spg 14 oog	8♂	7♂	7♂	XY to poles in 2nd	Wilson, '09 Wilson, '12	Jour. Exp. Zool., 6, p. 69 Jour. Exp. Zool., 13, p. 345
Lygaeus turcicus . .	14 spg 14 oog	8♂ (occasionally 9)	7♂	7♂	XY to poles in 2nd	Wilson, '05 Wilson, '06	Jour. Exp. Zool., 2, pp. 371 and 507 Jour. Exp. Zool., 3, p. 1

II. ARTHROPODA—Continued

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Oedancala dorsalis........	13 spg	7♂ (6 if small chroms. at poles)	7♂	6, 7♂	X to pole in 2nd	Montgomery, '01 Montgomery, '04 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Biol. Bull., 6, p. 137 Trans. Amer. Phil. Soc., 21, p. 97
Oncopeltus fasciatus.....	16 spg	9♂	8♂	8♂	XY to poles in 2nd	Montgomery, '01 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Trans. Amer. Phil. Soc., 21, p. 97
Oncopeltus fasciatus.....	16 spg 16 oog	9♂	8♂	8♂	XY to poles in 2nd (Sometimes X and Y equal in size)	Wilson, '09 Wilson, '12	Jour. Exp. Zool., 6, p. 69 Jour. Exp. Zool., 13, p. 345
Peliopelta abbreviata	14 spg	8♂ (9 if small chroms. side by side)	7♂		XY (equal) to poles in 2nd. From correction in '06	Montgomery, '01 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Trans. Amer. Phil. Soc., 21, p. 97
8. Nabidae							
Coriscus ferus............		10♂				Montgomery, '01	Trans. Amer. Phil. Soc., 20, p. 154
Nabis annulatus..........		10♂	8♂ (= 9, X and Y at poles in metaphase)	9♂	XY to poles in 2nd	Montgomery, '01 Montgomery, '06	Proc. Acad. Nat. Sci. Phila., 53, p. 261 Trans. Amer. Phil. Soc., 21, p. 97
9. Naucoridae							
Pelocoris femorata........	20? spg					Montgomery, '01	Trans. Amer. Phil. Soc., 20, p. 154
10. Notonectidae							
Notonecta glauca.........		12–13♂	12♂ (figured)			Pantel and Sinéty '06	La Cellule, 23, p. 89
Notonecta insulata.......		14♂	12, 13♂ (2 chroms. may be fused or not)	12♂	XY to poles in 2nd	Browne, '10 Browne, '13	Biol. Bull., 20, p. 19 Jour. Exp. Zool., 14, p. 61

Notonecta irrorata	24 spg	13♂	12♂	12♂	XY to poles in 2nd	Browne, '10 Browne, '13	Biol. Bull., 20, p. 19 Jour. Exp. Zool., 14, p. 61
Notonecta undulata	26 spg	14♂	13♂	13♂	XY to poles in 2nd	Browne, '10 Browne, '13	Biol. Bull., 20, p. 19 Jour. Exp. Zool., 14, p. 61
11. Pentatomidae							
Banasa calva	26 spg 26 oog	14♂	13♂	13♂	XY to poles in 2nd	Wilson, '05	Jour. Exp. Zool., 2, p. 507
		15♂	13, 14♂	13, 14♂	XY to poles in 2nd. S to pole in 1st. From correction in '07	Wilson, '07	Biol. Bull., 12, p. 303
Banasa dimidiata.........	16 spg 16 oog	9♂	8♂	8♂	XY to poles in 2nd	Wilson, '07	Biol. Bull., 12, p. 303
Brochymena sp?..........	14 spg	8♂	7♂	7♂	XY to poles in 2nd. From correction in '06	Montgomery, '01 Montgomery, '06	Trans. Amer. Phil Soc., 20, p. 154 Trans. Amer. Phil. Soc., 21, p. 97
Brochymena sp?.........	14 spg	8♂	7♂	7♂	XY to poles in 2nd	Wilson, '05	Jour. Exp. Zool., 2, p. 371
Coenus delius............	14 spg	8♂	7♂	7♂	XY to poles in 2nd. From correction in '06	Montgomery, '01 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Trans. Amer. Phil. Soc., 21, p. 97
Coenus delius............	14 spg 14 oog	8♂ (occasionally 9)	7♂	7♂	XY to poles in 2nd	Wilson, '05 Wilson, '06	Jour. Exp. Zool., 2, pp. 371 and 507 Jour. Exp. Zool., 3, p. 1
Cosmopepla carnifex......	16 spg	9♂	8♂	8♂	XY to poles in 2nd. From correction in '06	Montgomery, '01 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Trans. Amer. Phil. Soc., 21, p. 97
Eurygaster alternatus....		7♂	6♂	6♂	XY to poles in 2nd	Montgomery, '01 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Trans. Amer. Phil. Soc., 21, p. 97
Euschistus crassus........	12 spg 12 oog	7♂	6♂	6♂	XY to poles in 2nd	Foot and Strobell, '12	Arch. Zellf., 9, p. 47

II. ARTHROPODA—Continued

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Euschistus fissilis.........	14 spg 14 oog	8♂	7♂	7♂	XY to poles in 2nd	Wilson, '05 Wilson, '06	Jour. Exp. Zool., 2, pp. 371 and 507 Jour. Exp. Zool., 3, p. 1
Euschistus ictericus......	14 spg 14 oog				XY in spg. XX in oog	Wilson, '06	Jour. Exp. Zool., 3, p. 1
Euschistus servus........	14 spg 14 oog			7♂	XY to poles in 2nd	Wilson, '06	Jour. Exp. Zool., 3, p. 1
Euschistus servus........	14 som	8♂	7♂	7♂	XY to poles in 2nd	Foot and Strobell, '14	Arch. Zellf., 12, p. 485
Euschistus tristigmus.....	14 spg	8♂	7♂	7♂	XY to poles in 2nd	Montgomery, '01 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Trans. Amer. Phil. Soc., 21, p. 97
Euschistus tristigmus.....	14 spg 14 oog				XY in spg. XX in oog	Wilson, '06	Jour. Exp. Zool., 3, p. 1
Euschistus variolarius? (= "Pentatoma").	14 spg (13-15)	8♂ (8-11)	7♂ (6-9)	7♂	XY to poles in 2nd. From correction in '06. Prob. more than one species	Montgomery, '97 Montgomery, '98 Montgomery, '01 Montgomery, '06 Montgomery, '10 Montgomery, '11	Zool. Anz., 20, p. 457 Zool. Jahrb., 12, p. 1 Trans. Amer. Phil. Soc., 20, p. 154 Trans. Amer. Phil. Soc., 21, p. 97 Arch. Zellf., 5, p. 120 Jour. Morph., 22, p. 731
Euschistus variolarius....	14 spg 14 oog			7♂	XY to poles in 2nd	Wilson, '06	Jour. Exp. Zool., 3, p. 1
Euschistus variolarius....	14 som	8♂	7♂	7♂	XY to poles in 2nd	Foot and Strobell, '14	Arch. Zellf., 12, p. 485
Mineus bioculatus.......				7♂	XY to poles in 2nd. X and Y nearly equal	Wilson, '06	Jour. Exp. Zool., 3, p. 1
Mormidea lugens.........	14 spg	8♂	7♂		XY to poles in 2nd. From correction in '06	Montgomery, '01 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Trans Amer. Phil. Soc., 21, p. 97
Nezara hilaris...........	14 spg				From correction in '06	Montgomery, '01 Montgomery, '06	Trans. Amer Phil. Soc., 20, p 154 Trans. Amer. Phil. Soc., 21, p. 97

Nezara hilaris	14 spg 14 oog	8♂	7♂	7♂	XY to poles in 2nd. X and Y nearly equal	Wilson, '05	Jour. Exp. Zool., 2, p. 371
						Wilson, '06	Jour. Exp. Zool., 3, p. 1
						Wilson, '11	Jour. Morph., 22, p. 71
Nezara viridula	14 spg 14 oog	8♂	7♂	7♂	XY to poles in 2nd	Wilson, '05	Jour. Exp. Zool., 2, p. 371
						Wilson, '11	Jour. Morph., 22, p. 71
Oebalus pugnax .	10 spg 10 oog				XY in spg. XX in oog	Wilson, '09	Jour. Exp. Zool., 6, p. 69
Pentatoma juniperina (= Chlorochroa)	14 spg					Wilson, '13	Biol. Bull., 24, p. 392
Pentatoma senilis (= Rhytodoloma)	6 spg	4♂	3♂	3♂	XY to poles in 2nd	Wilson, '13	Biol. Bull., 24, p. 392
Pentatoma see Euschistus variolarius							
Peribalus limbolaris .	14 spg	8♂	7♂	7♂	XY to poles in 2nd. From correction in '06	Montgomery, '01	Trans. Amer. Phil. Soc., 20, p. 154
						Montgomery, '06	Trans. Amer. Phil. Soc., 21, p. 97
Perillus confluens .	14 spg	8♂	7♂	7♂	XY to poles in 2nd. From correction in '06	Montgomery, '01	Trans. Amer. Phil. Soc., 20, p. 154
						Montgomery, '06	Trans. Amer. Phil. Soc., 21, p. 97
Podisus bracteatus... Podisus crocatus	14 spg					Wilson, '09	Proc. 7th Intern. Zool. Cong., Boston, 1907, p. 1
Podisus modestus.... Podisus placidus..	16 spg					Wilson, '09	Proc. 7th Inter. Zool. Cong., Boston, 1907, p. 1
Podisus spinosus (= maculiventris) .	16 spg	9♂	8♂	8♂	XY to poles in 2nd. From correction in '06	Montgomery, '01	Trans. Amer. Phil. Soc., 20, p. 154
						Montgomery, '06	Trans. Amer. Phil. Soc., 21, p. 97 .
Podisus spinosus	16 spg 16 oog	9♂	8♂	8♂	XY to poles in 2nd	Wilson, '05	Jour. Exp. Zool., 2, p. 371
						Wilson, '06	Jour. Exp. Zool., 3, p. 1
Stiretrus anchorago	14 spg 14 oog				XY in spg. XX in oog	Wilson, '09	Jour. Exp. Zool., 6, p. 69

II. ARTHROPODA—Continued

FORM	DIPLOID AND PARTHENOGENETIC	1ST CYTE.	2ND CYTE.	TID	REMARKS	OBSERVER	REFERENCE
Thyanta calceata.........	27 spg 28 oog	15♂			XY to poles in 2nd X = 2 elements united linearly in 2nd	Wilson, '09 Wilson, '11	Proc. 7th Inter. Cong. Boston, 1907, p. 1 Jour. Morph., 22, p. 71
Thyanta custator.........	16 spg 16 oog				XY to poles in 2nd	Wilson, '11	Jour. Morph., 22, p. 71
Trichopepla semivittata..	16 spg	8♂	7♂	7♂	XY to poles in 2nd Small chroms. of diploid usually lacking in haploid	Montgomery, '01 Montgomery, '04 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Biol. Bull., 6, p. 137 Trans. Amer. Phil. Soc., 21, p. 97
Trichopepla semivittata..		8, 9♂	7♂		XY to poles in 2nd Small chrom. may be lacking in 1st (= S). Uncertain case	Wilson, '05	Jour. Exp. Zool., 2, p. 371
			12. Phymatidae				
Phymata wolffi?..........	29? spg	15♂			X? From correction in '06	Montgomery, '01 Montgomery, '06	Trans. Amer. Phil. Soc., 20, p. 154 Trans. Amer. Phil. Soc., 21, p. 97
			13. Pyrrochoridae				
Largus cinctus............	11 spg 12 oog				X in spg. XX in oog	Wilson, '07 Wilson, '09	Science, 25, no. 631, p. 191 Jour. Exp. Zool., 6, p. 69
Largus succintus.........	13 spg 14 oog				X in spg. XX in oog	Wilson, '07 Wilson, '09	Science, 25, no. 631, p. 191 Jour. Exp. Zool., 6, p. 69
Pyrrochoris apterus.......	24 spg 24 oog 24♀ som 24 cl	12♂ 12♀	12♂ 12♀	11, 12♂ 12♀	X to pole in 2nd♂ (= chromatin nucleolus)	Henking, '90 Henking, '91 Henking, '92	Intern. Monats. Anat. u. Phys., 7, p. 243 Zeits. wiss. Zool., 51, p. 685 Zeits. wiss. Zool., 54, p. 1

Pyrrochoris apterus..	24 spg 24 oog 24 som	12♂	12♂	11, 12♂ (those with 11 degenerate)	X to pole in 2nd. X = 2 elements, separate in diploid, united in haploid	Gross, '06 Gross, '12	Zool. Jahrb., 23, p. 269 Zool. Jahrb., Abt. f. Zool., 32, p. 99
Pyrrochoris apterus.......	23 spg 24 oog	12♂	12♂	11, 12♂	X to pole in 2nd	Wilson, '09	Jour. Exp. Zool., 6, p. 69
						Wilson, '09	Biol. Bull., 16, p. 199
			14. Reduviidae				
Acholla ampliata (= multispinosa)...........	32 spg	16♂	16♂			Montgomery, '01	Trans. Amer. Phil. Soc., 20, p. 154
						Montgomery, '06	Trans. Amer. Phil. Soc., 21, p. 97
						Montgomery, '10	Arch. Zellf., 5, p. 120 (note)
Acholla ampliata.......		16♂				Payne, '10	Biol. Bull., 18, p. 174
Acholla multispinosa (= Sinea diadema)		16♂	16♂		3 chroms associated in 1st	Montgomery, '01	Trans. Amer. Phil. Soc., 20, p. 154
						Montgomery, '06	Trans. Amer. Phil. Soc., 21, p. 97
						Montgomery, '10	Arch. Zellf., 5, p. 120 (note)
Acholla multispinosa.....	26 spg 30 oog	16♂	16♂	11, 15♂	XY to poles in 2nd X = 5 elements	Payne, '10	Biol. Bull., 18, p. 174
Apiomeris crassipes.......	24 spg	13♂	12♂	12♂	XY to poles in 2nd	Payne '12	Jour. Morph., 23, p. 331
Conorhinus sanguisugus	23 spg	13♂	13♂	11, 12♂	XY to poles in 2nd X = 2 elements	Payne, '09 Payne, '12	Biol. Bull., 16, p. 119 Jour. Morph., 23, p. 331
Diplocodus exsanguis.....	26♂ som 26♀ som	14♂	13♂	13♂	XY to poles in 2nd	Payne, '09	Biol. Bull., 16, p. 119
Fitchia spinosula.........	27 spg 28 oog	15♂	15♂	13, 14♂	XY to poles in 2nd X = 2 elements	Payne, '09	Biol. Bull., 16, p. 119
Pnirontis modesta........	25 spg	15♂	15♂	11, 14♂	XY to poles in 2nd X = 4 elements	Payne, '12	Jour. Morph., 23, p. 331

II. ARTHROPODA—Continued

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Prionidus cristatus	26 spg					Montgomery, '01 Montgomery, '06	Trans. Amer. Phil Soc., 20, p. 154 Trans Amer. Phil. Soc., 21, p. 97
Prionidus cristatus.......	26 spg 28 oog	15♂	15♂	12, 14♂	XY to poles in 2nd X = 3 elements	Payne, '09	Biol. Bull., 16, p. 119
Pselliodes cinctus.........	28 spg 30 oog	16♂	16♂	13, 15♂	XY to poles in 2nd X = 3 elements	Payne, '12	Jour. Morph., 23, p. 331
Reduvius personatus.....		12♂	11♂		XY to poles in 2nd	Payne, '12	Jour. Morph., 23, p. 331
Rocconata annulicornis...	27 spg	15♂	15♂	13, 14♂	XY to poles in 2nd X = 2 elements.	Payne, '09	Biol. Bull., 16, p. 119
Sinea complexa Sinea confusa Sinea diadema Sinea spinipes }	28 spg 30 oog	16♂	16♂	13, 15♂	XY to poles in 2nd X = 3 elements	Payne, '09 Payne, '12	Biol. Bull., 16, p. 119 Jour. Morph., 23, p. 331
Sinea rileyi...............		18♂	18♂	13, 17♂	XY to poles in 2nd X = 5 elements	Payne, '12	Jour. Morph., 23, p. 331
15. Tingitidae							
Tingis clavata............		7♂	7♂ (8 in one case, X and Y in same cell)	7♂	XY to poles in 1st	Montgomery, '01 Montgomery, '06	Proc. Acad. Nat. Sci. Phila., 53, p. 261 Trans. Amer. Phil. Soc., 21, p. 97
16. Homoptera							
1. Aphidae							
Aphid, bearberry = Phyllaphis coweni......	5 spg 6 oog	3♂. One cyst had 6 (2X)	2, 3♂. Cells with 2 degenerate. One cyst had 4, 6 or 5♂	3♂	X to pole in 1st. One cyst had 2X, to pole in 1st or one X to each pole	Morgan, '15	Jour. Exp. Zool., 19, p. 285

Aphid, beech (wooly).....		8♂	7, 8♂		X to pole in 1st. From correction in '09	Stevens, '06 Stevens, '09	Carnegie Inst. Pub., 51, p. 1 Jour. Exp. Zool., 6, p. 115
Aphid, birch, paper...		9♂				Stevens, '06	Carnegie Inst. Pub., 51, p. 1
Aphid, clover.......		8♂				Stevens, '06	Carnegie Inst. Pub., 51, p. 1
Aphid, goldenrod, beach..	12 pa cl	6♂	5, 6♂		X to pole in 1st. From correction in '09	Stevens, '06 Stevens, '09	Carnegie Inst. Pub., 51, p. 1 Jour. Exp. Zool., 6, p. 115
Aphid, goldenrod, tall = solidago altissima		6♂	5, 6♂		X to pole in 1st. From correction in '09	Stevens, '06 Stevens, '09	Carnegie Inst. Pub., 51, p. 1 Jour. Exp. Zool., 6, p. 115
Aphid, goumi............	10 cl (winter egg)	5♂		5♂ pron 5♀ pron		Stevens, '06	Carnegie Inst. Pub., 51, p. 1
Aphid, maple............		16♂	15, 16♂		X to pole in 1st	Stevens, '06 Stevens, '09	Carnegie Inst. Pub., 51, p. 1 Jour. Exp. Zool., 6, p. 115
Aphid, milkweed (black).	8 spg 8 pa cl	4♂ 8 pa ♀	3, 4♂		X to pole in 1st. From correction in '09	Stevens, '06 Stevens, '09	Carnegie Inst. Pub., 51, p. 1 Jour. Exp. Zool., 6, p. 115
Aphid, milkweed (orange)............... ..	8 pa cl	7, 8 pa ♀			May be ♂ and ♀ producing	Stevens, '06 Stevens, '09	Carnegie Inst. Pub., 51, p. 1 Jour. Exp. Zool., 6, p. 115
Aphid, milkweed (pale)..		7♂	7♂		One individual had 9 in 1st; probably different species	Stevens, '06	Carnegie Inst. Pub., 51, p. 1
Aphid, nasturtium.......		4♂	3, 4♂		May be black milkweed	Stevens, '06 Stevens, '09	Carnegie Inst Pub., 51, p. 1 Jour. Exp. Zool., 6, p. 115
Aphid, oak, red I.........		7♂	7♂			Stevens, '06	Carnegie Inst. Pub., 51, p. 1
Aphid, oak, red II.......		8♂				Stevens, '06	Carnegie Inst. Pub., 51, p. 1

II. ARTHROPODA—Continued

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Aphid, oak (white)........		7♂				Stevens, '06	Carnegie Inst. Pub., 51, p. 1
Aphid oenothera I = Aphis oenotherae	10 spg 10 pa cl 9♂ embryo	5♂ 5♀ 9♂ egg 10 pa ♀	4, 5♂	4, 5♂	X to pole in 1st. One chrom. goes to p.b., of ♂ egg. From correction in '09	Stevens, '05	Carnegie Inst. Pub., 36, p. 1
						Stevens, '05	Jour. Exp. Zool., 2, p. 313
						Stevens, '06	Carnegie Inst. Pub., 51, p. 1
						Stevens, '09	Jour. Exp. Zool., 6, p. 115
						Stevens, '10	Biol. Bull. 18, p. 72
Aphid, oenothera II......		4♂ 8 pa ♀	4♂	4♂		Stevens, '06	Carnegie Inst. Pub., 51, p. 1
Aphid, pea...............	8 pa cl	8 pa ♀				Stevens, '06	Carnegie Inst. Pub., 51, p. 1
Aphis rosae...............	8 + 3 double pa cl	14♀			One polar body	Stschelkanovzew, '04	Biol. Centr., 24, p. 104
Aphis rosae..............		10♀	10♀		One p.b. in pa eggs. Chroms unreduced	Hewitt, '06	Mem. and Proc. Manchester Lit. and Phil. Soc., 50, no. 6, p. 1
Aphis rosae = (brown) rose....................	10 spg 10 pa cl	5♂ 5♀ 10 pa ♀	5♂ 5♀		2 p.b.s. in winter egg and reduction. 1 p.b. in pa egg and no reduction	Stevens, '05	Jour. Exp. Zool., 2, p. 313
						Stevens, '06	Carnegie Inst. Pub., 51, p. 1
Aphis rosae..............	10 pa cl 10 pa som ♀	10♀				Von Baehr, '09	Arch. Zellf., 3, p. 269
Aphid, rose (green).......	14 pa cl	7♂ 14 pa ♀	6, 7♂		X to pole in 1st. From correction in '09	Stevens, '06	Carnegie Inst. Pub., 51, p. 1
						Stevens, '09	Jour. Exp. Zool, 6, p, 115
Aphid, rose (migratory)..	18 cl (winter egg)	9♀				Stevens, '06	Carnegie Inst. Pub., 51, p. 1

Aphis saliceti	5 spg 5♂ som 6♀ som 6 pa ♀ cl	3♂ 6♀. One case, 5	2, 3♂. Those with 2 degenerate	3♂	X to pole in 1st	Von Baehr, '08 Von Baehr, '09 Von Baehr, '12	Zool. Anz., 33, p. 507 Arch. Zellf., 3, p. 269 La Cellule, 17, p. 383
Aphis salicola............	5 spg	3♂	2, 3♂. Those with 2 degenerate	3♂	X to pole in 1st	Morgan, '09	Jour. Exp. Zool., 7, p. 239
Aphis salicola (= Harpswell willow)	5 spg 6 pa cl	3♂ 6 pa	4, 5♂		X to pole in 1st. From correction in '09	Stevens, '06 Stevens, '09	Carnegie Inst. Pub., 51, p. 1 Jour. Exp. Zool., 6, p. 115
Aphid, star cucumber		5♂	4, 5♂		X to pole in 1st From correction in '09	Stevens, '06 Stevens, '09	Carnegie Inst. Pub., 51, p. 1 Jour. Exp. Zool., 6, p. 115
Aphid, willow, Harpswell, see Aphis salicola							
Aphid, willow, Saranac...		5♂	4, 5♂		X to pole in 1st. From correction in '09	Stevens, '06 Stevens, '09	Carnegie Inst. Pub., 51, p. 1 Jour. Exp. Zool., 6, p. 115
Chaitophorus viminalis .		10♂				Morgan, '09	Jour. Exp. Zool., 7, p. 239
Lachnus dentatus . ..		4♂	3, 4♂. Those with 3 degenerate		X to pole in 1st	Morgan, '09	Jour. Exp. Zool., 7, p. 239
Melanoxanthus salicicola Melanoxanthus salicis	6 spg 6 oog 6 pa ♀ cl	3♂ 3♀ 6 pa ♀	3♂ 3♀	3♂ pron 3♀ pron	One p.b. in pa	Tannreuter, '07	Zool. Jahrb., 24, p. 609
Pemphigus pyriformis } .. Pemphigus spirothecae	20 pa ♀ som	20♀				Von Baehr, '08 Von Baehr, '09	Zool. Anz., 33, p. 507 Arch. Zellf., 3, p. 269
Phyllaphis coweni, see Aphid, bearberry							
Phylloxera caryaecaulis ..	5(= 6) spg 6(= 8) oog	3(= 4)♂ 3(= 4)♀ 6(= 8)♀ pa	3(= 4), 2♂ 3(= 4)♀ Spc with 2 degenerate	3(= 4)♂ 3(= 4)♀ 5♂ pa egg 6♀ pa egg	Double X to pole in 1st. Double X to p.b. in pa ♂ producers. One p.b. in pa line.	Morgan, '08 Morgan, '09 Morgan, '09 Morgan, '12 Morgan, '15	Proc. Soc. Exp. Biol. and Med., 5, p. 56 Science, 29, p. 234 Jour. Exp. Zool., 7, p. 239 Jour. Exp. Zool., 12, p. 479 Jour. Exp. Zool., 19, p. 285

II. ARTHROPODA—Continued

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Phylloxera caryaefoliae…	8 egg of migrant					Morgan, '09	Jour. Exp. Zool., 7, p. 239
Phylloxera caryaeglobuli.	22♂ eggs 22♀ eggs					Morgan, '06 Morgan, '09	Biol. Bull., 10, p. 201 Jour. Exp. Zool., 7, p. 239
Phylloxera depressa……	6 egg of stem mother					Morgan, '09	Jour. Exp. Zool., 7, p 239
Phylloxera fallax………	10 spg 10 oog 10♀ som	6♂ 6♀ 12 pa♀	4, 6♂ 6♀ Spc with 4 degenerate	6♂ 6♀ 10♂ pa egg 12♀ pa egg	2X to pole in 1st; may be united. 2X to p.b. in pa ♂ producers. One p.b. in pa line	Morgan, '06 Morgan, '09 Morgan, '09 Morgan, '12 Morgan, '15	Biol. Bull., 10, p. 201 Science, 29, p. 234 Jour. Exp. Zool., 7, p. 239 Jour. Exp. Zool., 12, p. 479 Jour. Exp. Zool., 19, p. 285
Phylloxera globosum…..	6 egg of stem mother 6♂ eggs 6♀ eggs					Morgan, '06 Morgan, '09	Biol. Bull., 10, p. 201 Jour. Exp. Zool., 7, p. 239
Phylloxera subelliptica…	6 egg of migrant					Morgan, '09	Jour. Exp. Zool., 7, p. 239
Schizoneura lanigera } Schizoneura ulmi } ….	12 pa♀ som	12♀ (11 in 2 cases)				Von Baehr, '08 Von Baehr, '09	Zool. Anz., 33, p. 507 Arch. Zellf., 3, p. 269
				2. Cercopidae			
Aphrophora parallela…..		15♂	14, 15♂		X to pole in 1st	Boring and Fogler, '15	Biol. Bull., 29, p. 312
Aphrophora quadrangularis (= Lepronia quad.)………………	21 spg	11♂ 12♂	10, 11♂ 11, 12♂	10, 11♂	X to pole in 1st. Probably 2 species	Boring, '07 Boring, '13	Jour. Exp. Zool., 4, p. 469 Biol. Bull., 24, p. 133
Aphrophora quadrangularis (Harpswell)…….	23 spg 24 oog 23♂ som	12♂	11, 12♂	11, 12♂	X to pole in 1st	Stevens, '06	Carnegie Inst. Pub. no. 36, II, p. 33

Aphrophora quadrinotata		14♂	13, 14♂	13, 14♂	X to pole in 1st	Boring, '07	Jour. Exp. Zool., 4, p. 469
Aphrophora spumaria....		6–12♂				Carnoy, '85	La Cellule, 1, p. 189
Aphrophora spumaria....		12♂			X to pole in 1st	Boring, '13	Biol. Bull., 24, p. 133
Clastoptera obtusa.......	15 spg	8♂	7, 8♂	7, 8♂	X to pole in 1st	Boring, '07	Jour. Exp. Zool., 4, p. 469
Clastoptera proteus.......		7♂			X to pole in 1st	Boring and Fogler, '15	Biol. Bull., 29, p. 312
Lepronia quadrangularis, see Aphrophora quadrangularis							
Philaenus lineatus........	29 spg	15♂	14, 15♂		X to pole in 1st	Boring and Fogler, '15	Biol. Bull., 29, p. 312
Philaenus spumarius.....	23 spg 24 oog 23♂ som 24♀ som	12♂	11, 12♂		X to pole in 1st	Boring, '13	Biol. Bull., 24, p. 133
			3. Cicadidae				
Cicada tibicen............	12 spg	6♂ (= 24 el)				Wilcox, '95	Bull. Mus.Comp. Zool. Harvard, 27, p. 3
			4. Coccidae				
Icerya purchasi...........	4 spg 4 oog 4 som	2♂ 2♀	2♂ 2♀			Pierantoni, '12 Pierantoni, '14	Archivio Zoologico, 5, p. 321 Archivio Zoologico, 7, p. 27
			5. Fulgoridae				
Amphiscepa bivittata....	25 spg	13♂	12, 13♂	12, 13♂	X to pole in 1st	Boring, '07	Jour. Exp. Zool., 4, p. 469
Poeciloptera bivittata....		13♂	12, 13♂	12, 13♂	X to pole in 1st	Boring, '07	Jour. Exp. Zool., 4, p. 469
Poeciloptera pruinosa Poeciloptera septentrionalis	27 spg 28♀ som	14♂	13, 14♂	13, 14♂	X to pole in 1st	Boring, '07	Jour. Exp. Zool., 4, p. 469

II. ARTHROPODA—Continued

6. Jassidae

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Agallia sanguinolenta.....		11♂	10, 11♂	10, 11♂	X to pole in 1st	Boring, '07	Jour. Exp. Zool., 4, p. 469
Chlorotettix unicolor Chlorotettix viridus.....}	21 spg	11♂ 9♂	10, 11♂ 8, 9♂	10, 11♂ 8, 9♂	X to pole in 1st Two species confused	Boring, '07	Jour. Exp. Zool., 4, p. 469
Diedrocephala coccinea. Diedrocephala mollipes. }	23 spg	12♂	11, 12♂	11, 12♂	X to pole in 1st	Boring, '07	Jour. Exp. Zool., 4, p. 469
Phlepsius irroratus.......	15 spg	8♂	7, 8♂	7, 8♂	X to pole in 1st	Boring, '07	Jour. Exp. Zool., 4, p. 469

7. Membracidae

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Atymna castanea.........		11♂	10, 11♂	10, 11♂	X to pole in 1st	Boring, '07	Jour. Exp. Zool., 4, p. 469
Campylonchia curvata...	19 spg	10♂	9, 10♂	9, 10♂	X to pole in 1st	Boring, '07	Jour. Exp. Zool., 4, p. 469
Ceresa bubalus Ceresa diceros Ceresa taurina }		11♂	10, 11♂	10, 11♂	X to pole in 1st	Boring, '07	Jour. Exp. Zool., 4, p. 469
Enchenopa binottata.....	19 spg (20 in 2 cases)	10♂	10♂	9, 10♂	X to pole in 2nd	Boring, '07	Jour. Exp. Zool., 4, p. 469
Enchenopa binottata .	20 spg 20 oog	10♂	10♂	10♂	XY to poles in 1st	Kornhauser, '14	Arch. Zellf., 12, p. 241 or Bull. Mus. Comp. Zool., Harvard, 244, p. 241
Enchenopa curvata (= Campylenchia curvata)	19 spg 20 oog	10♂	9, 10♂ (occasionally 19, no cell division)	9, 10♂	X to pole in 1st. Occasionally X divides	Kornhauser, '14	Arch. Zellf., 12, p. 241 or Bull. Mus. Comp. Zool. Harvard, 244, p. 241
Entilia sinuata......	21 spg	11♂	10, 11♂	10, 11♂	X to pole in 1st	Boring, '07	Jour. Exp. Zool., 4, p. 469

Thelia bimaculata........	21 spg 22 oog	11♂			X to pole in 1st	Kornhauser, '14	Arch. Zellf., 12, p. 241 or Bull. Mus. Comp. Zool. Harvard, 244, p. 241
Vanduzea arcuata........	17 spg	9♂	8, 9♂	8, 9♂	X to pole in 1st	Boring, '07	Jour. Exp. Zool., 4, p. 469
				5. Hymenoptera a. Apidae			
Apis mellifica (= "Biene")...........	16 pa cl	16♀	8♀	8♀	Drone egg pa, with 2 p.b.s. Ootid chroms probably split in cl	Petrunkewitsch., '01 Petrunkewitsch., '03	Zool. Jahrb., 14, p. 573 Zool. Jahrb., 17, p. 481
Apis mellifica............	16 spg	16♂	16♂	16♂	Half 1st spc rudimentary with no chroms. Half 2nd spc rudimentary with chroms	Meves, '03 Meves, '07	Anat. Anz., 24, p. 29 Arch. mikr. Anat., 70, p. 414
Apis mellifica (= "Honey-bee")		16♂	16♂		As above	Mark and Copeland, '06	Proc. Amer. Acad. Arts and Sci., 42, p. 101
Apis mellifica............	16 oog	8♂ (double)	16♂	16♂	As above	Doncaster, '06 Doncaster, '07	Anat. Anz., 29, p. 490 Anat. Anz., 31, p. 168
Apis mellifica	16 spg (single) 16 oog (double) 16 pa cl 32 fert. cl	16♂ (dyads) 8♀ (tetrads)	16♂ (dydas) 8♀ (dyads)	16♂ (8 double) 8♀ (bivalent) 16♀ pron	As above	Nachtsheim, '12 Nachtsheim, '13	Sitz. Gesel. Morph. u. Physiol. München, '12, p. 1 Arch. Zellf., 11, p. 169
Osmia cornuta............	16 spg 32 or 64 som	16♂		8♂	Chroms pair at 1st anaphase. 1 mat. div. and half 1st spc rudimentary with chroms	Armbruster, '13 Armbruster, '13	Ber. Naturf. Ges. Freiburg, 20, p. 4 Arch. Zellf., 11, p. 242
Xylocopa violacea........	16 spg Numerous in follicle cells	16♂	16♂	16♂	As above in Apis	Granata, '09 Granata, '13	Biologica 2, no. 15, p. 1 Mon. Zool. Ital., 24, p. 31
				b. Chalcididae			
Ageniaspis fuscicollis (= Encyrtus)		ca 10♀			2 p.b.s in pa. Chroms fuse later	Silvestri, '08	Bol. R. Scuola Sup. Portici, 3, p. 29

II. ARTHROPODA—Continued

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Ageniaspis fuscicollis.....		4♀	4♀	4♀		Martin, '14	Zeit. wiss. Zool., 110, p. 419
Copidosoma buyssoni....		12♀	10–12♀			Silvestri, '14	Anat. Anz., 47, p. 45
Copidosoma gelechiae....		12♀ Sometimes 11			May not be mat. spindle	Hegner, '14 Hegner, '15	Anat. Anz., 46, p. 51 Jour. Morph., 26, p. 495
Prospalta berlesei (= Prospaltella)...........		ca 10–12♀			1 p.b.	Silvestri, '08 Silvestri, '15	Boll. Lab. Zool. R. Sc., Agr. Portici, 3, p. 22 Boll. Lab. Zool. R. Sc., Agr. Portici, 10, p. 64
c. Cynipidae							
Dryophanta erinacei......	12 som♂ 13–14 som♀		12♂		1st spc div. = cytop. only	Wieman, '15	Biol. Bull., 28, p. 34
Neuoterus lenticularis (= Spathegaster baccarum)........	10 pa cl 10 som♂ 20 pa cl 20 som♀	10♀	10♀	10♀	Parthenogenetic. 2 types of eggs, one with p.b.s. may be ♀s; other lacking p.b.s. ♂s	Doncaster, '09 Doncaster, '11	Proc. Roy. Soc., 82B, p. 88 Proc. Roy. Soc. 83B, p. 476
	10 spg 20 oog 18–20 cl 18–20 som	10♀	10♂	10♂ 10? ♀	Sexual. Half 1st spc rudimentary with no chroms		
Rhodites rosae............	18 pa cl (= 18–20)	9♀	9♀			Henking, '02	Zeit. wiss. Zool., 54, p. 1
Rhodites rosae............	12 oog 12 pa cl, later = 6 double	10–12♀ (prob. 12)	10–12♀	10–12♀		Schleip, '09	Zool. Anz., 35, p. 203
d. Formicidae							
Formica sanguinea.......	ca 24 pa cl ca 48 fert. cl	24♀	24♀	24♀	Pa and fert.	Schleip, '08	Zool. Jahrb., 26, p. 651
Lasius niger..............	16–17 pa cl 20 fert. cl	10♀	10♀			Henking, '92	Zeit. wiss. Zool., 54, p. 1

e. Tenthredinidae							
Croesus varus			7-8♀, split later into 14-16		Parthenogenetic	Doncaster, '06	Q. J. M. S., 49, p. 561
Nematus lacteus			ca 8♀		Parthenogenetic	Doncaster, '06	Q. J. M. S., 49, p. 561
Nematus ribesii	ca 16 spg	ca 8 ♂ ca 8♀			1st spc div. abortive. Eggs parthenogenetic. From correction in '09	Doncaster, '04 Doncaster, '06 Doncaster, '07 Doncaster, '08 Doncaster, '09 Doncaster, '10	Proc. Camb. Phil. Soc., 12, p. 474 Q. J. M. S., 49, p. 561 Q. J. M. S., 51, p. 101 Proc. Camb. Phil. Soc., 14, p. 22 Nature 82, p. 127 Science, 31, p. 192
Poecilosoma luteolum	8 pa cl	8♀ (possibly 7)			Parthenogenetic	Doncaster, '06 Doncaster, '06	Proc. Camb. Phil. Soc., 13, p. 103 Q. J. M. S., 49, p. 561
f. Vespidae							
Vespa crabro		16+♀			1st spc rudimentary with no chroms	Meves and Duesberg, '08	Arch. mikr. Anat., 71, p. 571
Vespa maculata		16?♂				Mark and Copeland, '07	Proc. Amer. Acad. Arts and Sci., 43, p. 69
C. *Lepidoptera* a. Heterocera 1. Arctiidae							
Arctia caja		31♂	31♂			Kernewitz, '14 Kernewitz, '15	Zool.. Anz., 45, p. 137 Arch. Naturges., 81, p. 1
Arctia hebe		30-33♂				Kernewitz, '14 Kernewitz, '15	Zool. Anz., 45, p. 137 Arch. Naturges., 81, p. 1
Chelonia caja		24-28♂				Carnoy, '85	La Cellule, 1, p. 189
Hipocrita jacobaea		31♂				Kernewitz, '14 Kernewitz, '15	Zool. Anz., 45, p. 137 Arch. Naturges., 81, p. 1

II. ARTHROPODA—Continued

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Phragmatobia fuliginosa	prob. 56 spg 56♂ cl 58♀ cl Few 61, 62 cl	28♂ 28♀	28♂ 28, 29♀	28♂ 28, 29♀	XX in ♂ diploid. X + 3Y in ♀ diploid. XY in ♀ to poles in 1st. Y splits in two in 1st anaphase = 29	Seiler, '13 Seiler, '14	Zool. Anz., 41, p. 246 Arch. Zellf., 13, p. 159
Spilosoma mendicum.....		31♂	31♂			Kernewitz, '14 Kernewitz., '15	Zool. Anz., 45, p. 137 Arch. Naturges., 81, p. 1
			2. Bombycidae				
Bombyx mori............		12♀ at least				Henking, '92	Zeit. wiss. Zool., 54, p. 1
Bombyx mori	26–28 spg	28♂(26–28)	28♂	14♂	Half chroms to each pole in 2nd	Toyama, '94 Toyama, '94	Zool. Anz., 17, p. 20 Imp. Univ. Tokyo Coll. Agr. Bull., vol. II, no. 3, p. 125
Bombyx mori (17 varieties)....................	50–60 spg 50–60 oog	28♂	28♂	28♂		Yatsu, '13	Annot. Zool. Japonenses, 8, pt. 2, p. 215
Theophila mandriana (= Bombyx)...........		27♂	27♂			Yatsu, '13	Annot. Zool., Japonenses, 8, pt. 2, p. 215
			3. Geometridae				
Abraxas grossulariata Abraxas lacticolor and cross	56 spg (50–56) 56 oog 55 oog in some strains	28♂ (1 case 27) 28♀	28♂ or 26 + 1 pair in contact 28♀ in 56 strains 27, 28♀ in 55 strains	28♂ 28♀ 27, 28♀	XX in ♂ diploid. X in ♀ diploid. X to pole in 2nd ♀	Doncaster, '10 Doncaster, '11 Doncaster, '12 Doncaster, '13 Doncaster, '14	Proc. Camb. Phil. Soc. 16, p. 44 Jour. Genetics 1, p. 179 Jour. Genetics, 2, p. 189 Jour. Genetics, 3, pp. 1 and 229 Jour. Genetics, 4, p. 1
Nyssia zonaria (= Ithysia)......................	ca 112 spg (100+)	56♂ 50–60♀	56♂			Doncaster, '14	Jour. Genetics, 4, p. 1
Ourapteryx sambucaria...		31♂				Kernewitz, '14 Kernewitz, '15	Zool. Anz., 45, p. 137 Arch. Naturges., 81, p. 1

4. Lasiocampidae

Cosmotriche potatoria		31♂				Kernewitz, '14 Kernewitz, '15	Zool. Anz., 45, p. 137 Arch. Naturges., 81, p. 1
Dendrolimus pini........		30♂	30♂			Kernewitz, '14 Kernewitz, '15	Zool. Anz., 45, p. 137 Arch. Naturges., 81, p. 1
Malacosoma castrense.....		31♂				Kernewitz, '15	Arch. Naturges., 81, p. 1

5. Lymantriidae

Leucoma salicis...........		12+♀				Henking, '92	Zeit. wiss. Zool., 54, p. 1
Lymantria dispar Lymantria japonica }	62 cl	31♂ 31	31♂	31♂	One embryo with over 100	Seiler, '14	Arch. Zellf., 13, p. 159
Orgyia antiqua...........		14♀				Seiler, '14	Arch. Zellf., 13, p. 159
Orgyia gonostigma........		30♀				Seiler, '14	Arch. Zellf., 13, p. 159

6. Monocteniidae

Lycia hirtaria (= Biston)	28 spg 28 oog	13♂ (1 double) 13 + chrom. nucleolus ♀	13 (1 double), 14♂		Possibly X to pole in 1st	Doncaster, '14	Jour. Genetics, 3, p. 229

7. Noctuidae

Acronycta sp?............		29♂				Cook, '10	Proc. Acad. Nat. Sci. Phila., 62, p. 294
Agrostis triangulum......		29♂	29♂			Kernewitz, '14 Kernewitz, '15	Zool. Anz., 45, p. 137 Arch. Naturges., 81, p. 1
Leucania impura.........		31♂	31♂			Kernewitz, '14 Kernewitz, '15	Zool. Anz., 45, p. 137 Arch. Naturges., 81, p. 1

8. Notodontidae

Dicranura vinula.........		21♂				Kernewitz, '14 Kernewitz, '15	Zool. Anz., 45, p. 137 Arch. Naturges., 81, p.1

II. ARTHROPODA—Continued

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST CYTE	2ND CYTE	TID	REMARKS	OBSERVER	REFERENCE
Phalera bucephala........	ca 60 spg					Kernewitz, '15	Arch. Naturges., 81, p. 1
Pygaera anchoreta........		30♂ (sometimes 31)	30♂		Different in apyrene	Federley, '13	Zeit. ind. Abs. u. Vererb., 9, p. 1
Pygaera bucephala		30♂	12-14♂			Platner, '89	Intern. Monats. Anat. u. Physiol., 3, p. 341
Pygaera curtula..........		29♂	29♂			Federley, '13	Zeit. ind. Abs. u. Vererb., 9, p. 1
Pygaera pigra	40+ spg	23♂	23♂		Different in apyrene	Federley, '13	Zeit. Ind. Abs. u. Vererb., 9, p. 1
9. Pyralidae							
Ephestia kuehniella......		29♂	29♂			Kernewitz, '15	Arch. Naturges., 81, p. 1
Galleria melonella........	ca 60 spg	30♂			Different in apyrene	Von Kemnitz, '14	Arch. Zellf., 12, p. 567
10. Saturnidae							
Antherea pernyi..........			33♂ (sometimes 34)			Kernewitz, '14 Kernewitz, '15	Zool. Anz., 45, p. 137 Arch. Naturges., 81, p. 1
Automeris io............		31♂	31♂		Equal XY	Cook, '10	Proc. Acad. Nat. Sci., Phila., 64, p. 294
Callosamia promethea....	38 spg	19♂	19♂	19♂	Equal XY	Cook, '10	Proc. Acad. Nat. Sci., Phila., 64, p. 294
Philosamia cynthia.......	26 spg 26 oog 26 som	13♂ 13♀	13♂ 13♀	13♂ 13♀		Dederer, '07 Dederer, '15	Biol. Bull., 13, p. 94 Jour. Morph., 26, p. 1
Philosamia cynthia	26 spg	13♂				Cook, '10	Proc. Acad. Nat. Sci., Phila., 64, p. 294
Samia cecropia...........		30♂	30♂		Equal XY	Cook, '10	Proc. Acad. Nat. Sci., Phila., 64, p. 294

Saturnia pavonia.........		29♂	29♂			Kernewitz, '15	Arch. Naturges., 81, p. 1
Telea polyphemus........		30♂	30♂			Cook, '10	Proc. Acad. Nat. Sci. Phila., 64, p. 294
11 Sphingidae							
Cherocampa elpenor......		29♂	29♂			Kernewitz, '14 Kernewitz, '15	Zool. Anz., 45, p. 137 Arch. Naturges., 81, p. 1
Deilephila euphorbiae....		28–29♂				Kernewitz, '14 Kernewitz, '15	Zool. Anz., 45, p. 137 Arch. Naturges., 81, p. 1
Deilephila euphorbiae.	28 spg	28♂ (sometimes 29)	28♂	14♂	Reduction in 2nd	Buder, '15	Arch. Zellf., 14, p. 26
Dilina tiliae...		29♂				Federley, '14	Ofver. Finsk. Vetensk. Soc. Förhand., 56, p. 1
Dilina tiliae.......... .		29♂				Kernewitz '15	Arch. Naturges., 81, p. 1
Smerinthus ocellatus		27♂	27♂			Federley, '14	Ofver. Finsk. Vetensk. Soc. Förhand., 56, p. 1
Smerinthus ocellatus.....		28♂	28♂			Kernewitz, '15	Arch. Naturges., 81, p. 1
Smerinthus populi........		28♂	28♂			Federley, '14	Ofver. Finsk. Vetensk. Soc. Förhand., 56, p. 1
Sphinx ligustri...........		27–29♂				Kernewitz, '14 Kernewitz, '15	Zool. Anz., 45, p. 137 Arch. Naturges., 81, p. 1
12 Tortricidae							
Cacaecia cerasivorana.....		30♂	30♂		Equal XY	Stevens, '06	Carnegie Inst. Pub., 36, II, p. 33

II. ARTHROPODA—Continued

b. Rhopalocera

1. Nymphalidae

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Euvanessa antiope........		30♂			Equal XY	Stevens, '06	Carnegie Inst. Pub., 36, II, p. 33
Polygonia-c-album.......			31♂			Kernewitz, '14 Kernewitz, '15	Zool. Anz., 45, p. 137 Arch. Naturges., 81, p. 1
2. Papilionidae							
Papilio podalirius	54-58 spg					Kernewitz, '15	Arch. Naturges., 81, p. 1
Papilio rutulus...........	28 spg	28♂	28♂	14♂		Munson, '06	Proc. Boston Soc. Nat. Hist., 33, p. 43
3. Pieridae							
Aporia crataegi			25♂			Kernewitz, '14 Kernewitz, '15	Zool. Anz., 45, p. 137 Arch. Naturges., 81, p. 1
Colias myrmidone . .			30-31♂			Kernewitz, '15	Arch. Naturges., 81, p. 1
Pieris brassicae.	ca 30 spg ca 28 oog ca 28 cl	14 or 15♂ 14♀	14♀	14♀		Henking, '90 Henking, '90 Henking, '91 Henking, '92	Intern. Monats. Anat. u. Physiol., 7, p. 243 Zeits. wiss. Zool., 49, p. 503 Zeits. wiss. Zool., 51, p. 685 Zeits. wiss. Zool., 54, p. 1
Pieris brassicae...........	ca 30 spg 30 oog	15♂ 15♀ (= 14 + chromatin nucleolus) One cell 25	15♂		Possibly XY in ♂ No XY in ♀	Doncaster, '12 Doncaster, '12	Proc. Camb. Phil. Soc., 16, p. 491 Jour. Genetics, 2, p. 189
Pieris napi...............	50? spg	25♂				Henking, '90	Intern. Monats. Anat. u. Physiol., 7, p. 243

7. *Neuroptera*

a. Corrodentia

Cerastipsocus venosus	17 spg	9♂	8, 9♂	8, 9♂	X to pole in 1st	Boring, '13	Biol. Bull., 24, p. 125
b. Isoptera							
Termopsis angusticollis...	52 spg	26♂	26♂		No X	Stevens, '05	Carnegie Inst. Pub., 36, p. 1
c. Odontata							
Anax junius.............	27 spg 28 follicle cells	14♂	14♂	13, 14♂	X to pole in 2nd. From correction in '08	McGill, '04 Lefevre and McGill, '08	Univ. Missouri Studies, 2, no. 5, p. 1 Amer. Jour. Anat., 7, p. 469
Calopteryx virgo.........		8-14♂	5-7♂			Carnoy, '85	La Cellule, 1, p. 189
d. Trichoptera							
Panorpa communis.......		14-18♂				Carnoy, '85	La Cellule, 1, p. 189
Platyphlax designatus....	55-60 oog	30♂	30♂		Chromatin nucleolus divides in both divisions	Lutman, '10	Biol. Bull., 19, p. 55
8. *Orthoptera* a. Acrididae							
Acridium granulatus see under Tettigidae Acridium linolea..........		10-16♂				Carnoy, '85	La Cellule, 1, p. 189
Acrolophitus.............	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Acoloplus	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Amphitornus............	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Arphia..................	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Arphia pseudonietana....	23 spg					Meek, '13	Phil. Trans. Roy. Soc., London 203B, p. 1

II. ARTHROPODA—Continued

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	TID	REMARKS	OBSERVER	REFERENCE
Arphia simplex	23 spg	12♂			X to pole in 1st	Carothers, '13	Jour. Morph., 24, p. 487
Arphia tenebrosa	23 spg	12♂	11, 12♂	11, 12♂	X to pole in 1st. One animal had 2 X, to same or opposite poles in 1st; = 24 spg, 13 1st, 11, 12, 13 2nd	Davis, '08	Bull. Mus. Comp. Zool. Harvard, 53, p. 57
Aulocara	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Boopedon	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Brachystola	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Brachystola magna	23 spg 22 oog	12♂	11, 12♂	11, 12♂	X to pole in 1st	Sutton, '02	Biol. Bull., 4, p. 24
Brachystola magna	23 spg	12♂		11, 12♂	X to pole in 1st	Carothers, '13	Jour. Morph., 24, p. 487
Caloptenus femur-rubrum, see Melanoplus							
Camnula	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Chortophaga viridifasciata	19 spg (= 23) or 23 spg	12♂	11, 12♂		X to pole in 1st. Union of chroms. in spg to form tetrads	McClung, '05 McClung, '14	Biol. Bull., 9, p. 304 Jour. Morph., 25, p. 651
Chortophaga viridifasciata	23 spg	12♂	11, 12♂	11, 12♂	X to pole in 1st	Davis, '08	Bull. Mus. Comp. Zool. Harvard, 53, p. 57
Clinocephalus	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Dactylotum	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651

Dissosteira	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Dissosteira carolina	23 spg	12♂	11, 12♂	11, 12♂	X to pole in 1st	Davis, '08	Bull. Mus. Comp. Zool. Harvard, 53, p. 57
Encoptolophus	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Eremnus	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Hadrotettix	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Hesperotettix pratensis Hesperotettix speciosus Hesperotettix viridis	22 spg (= 23)	11♂ (= 12)			X attached to another chromosome in spg and 1st. To pole in 1st	McClung, '05 McClung, '14	Biol. Bull., 9, p. 304 Jour. Morph., 25, p. 651
Hippiscus phoenicopterus	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Hippiscus tuberculatus...	23 spg 24 oog	12♂	11, 12♂	11, 12♂	X to pole in 1st	Davis, '08	Bull. Mus. Comp. Zool. Harvard, 53, p. 57
Mecostethus .	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Melanoplus	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Melanoplus angustipennis	23 spg		11, 12♂		X	Meek, '13	Phil. Trans Roy. Soc., London, 203B, p. 1
Melanoplus atlanis .	23 spg	12♂	11, 12♂		X to pole in 1st	Nowlin, '12	Kansas Univ. Sci. Bull., 6, p. 309
Melanoplus atlanis .	23 spg		11, 12♂		X	Meek, '13	Phil. Trans. Roy. Soc. London, 203B, p. 1
Melanoplus bivittatus	23 spg	12♂	11, 12♂		X to pole in 1st	Nowlin, '08	Kansas Univ. Sci. Bull., 4, p. 265
Melanoplus bivittatus	23 spg					Meek, '13	Phil. Trans. Roy. Soc. London, 203B, p. 1
Melanoplus dawsoni .	23 spg					Meek, '13	Phil. Trans. Roy. Soc. London, 203B, p. 1

II. ARTHROPODA—Continued

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Melanoplus differentialis..	23 spg	12♂	11, 12♂		X to pole in 1st	Nowlin, '12	Kansas Univ. Sci. Bull., 6, p. 399
Melanoplus femoratus....	23 spg	12♂	11, 12♂	11, 12♂	X to pole in 1st	Davis, '08	Bull. Mus. Comp. Zool. Harvard, 53, p. 57
Melanoplus femur-rubrum (= Caloptenus)....	12 spg 12 som	6♂ (= 24 el)	6♂ (= 12 el)	6♂	Tetrads and dyads = separate elements	Wilcox, '94 Wilcox, '95 Wilcox, '98	Anat. Anz., 10, p. 303. Bull. Mus. Comp. Zool., Harvard, 27, p. 3 Anat. Anz., 14, p. 194
Melanoplus femur-rubrum....................	23 spg 22? oog	12♂	11, 12♂	11, 12♂	X to pole in 1st	Nowlin, '12	Kansas Univ. Sci. Bull., 6, p. 399
Melanoplus packardii....	23 spg	12♂	11, 12♂		X to pole in 1st	Nowlin, '12	Kansas Univ. Sci. Bull., 6, p. 399
Melanoplus packardii.....	23 spg		11, 12♂		X	Meek, '13	Phil. Trans. Roy. Soc. London, 203B, p. 1
Mermiria bivittata........	21 spg (= 23)	10♂ (= 12)			X attached to two other chromos., one of which passes to each pole in 1st, X going to pole with one of them	McClung, '05 McClung, '14	Biol. Bull., 9. p. 304 Jour. Morph., 25, p. 651
Mestobregma	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Oedipoda.................	23 spg	12♂	11, 12♂	11, 12♂	X to pole in 1st	Buchner, '09	Arch. Zellf., 3, p. 335
Orphulella................	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Pamphagus marmoratus..	19 spg 20♀ som	10♂			X to pole in 1st	Granata, '10	Arch. Zellf., 5, p. 182
Paratettix, see under Tettigidae							
Paroxya..................	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651

Philbostroma	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Phoetaliotes............	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Phrynotettix	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Phrynotettix magnus	23 spg	12♂	11, 12♂		X to pole in 1st	Pinney, '08	Kansas Univ. Sci. Bull., 4, p. 309
Proracorypha	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Pseudopomala	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Psinidia	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Rhomaleum	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Schistocerca	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Schistocerca alutacea Schistocerca americana }	23 spg	12♂				Hartmann, '13	Biol. Bull., 24, pp. 226 and 239
Scirtettica	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Spharagemon	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Stauronotus maroccanus	23 spg	12♂	11, 12♂	11, 12♂	X to pole in 1st	Artom, '09	Biologica, 2, no. 16, p. 1
Stenobothrus (prob. = Chorthippus).. .	21 spg	11♂	10, 11♂		X to pole in 1st	McClung,' 14	Jour. Morph., 25, p. 651
Stenobothrus bicolor.	17 spg	9♂	8, 9♂		X to pole in 1st	Meek, '13	Phil. Trans. Roy. Soc., London, 203B, p. 1
Stenobothrus biguttulus	16–17 spg (prob. 17)	9♂	8, 9♂	8, 9♂	X to pole in 1st	Gérard, '09	Bull. Soc. Roy. Sci. med. et nat. Bruxelles, 67, p. 25
						Gérard, '09	Arch. Biol., 24, p. 543
Stenobothrus curtipennis . . .	17 spg	9♂	8, 9♂	8, 9♂	X to pole in 1st	Davis, '08	Bull. Mus. Comp. Zool. Harvard, 53, p. 57

II. ARTHROPODA—Continued

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Stenobothrus curtipennis	17 spg	9♂	8, 9♂			Meek, '12	Jour. Linnean Soc., 32, p. 107
Stenobothrus parallelus	17 spg		8, 9♂		X to pole in 1st	Meek, '13	Phil. Trans. Roy. Soc. London, 203B, p. 1
Stenobothrus viridulus (or bicolor)		12-18♂	6-8♂			Carnoy, '85	La Cellule, 1, p. 189
Stenobothrus viridulus	17 spg	9♂	8, 9♂		X to pole in 1st	Meek, '11 Meek, '13	Jour. Linnean Soc., 32, p. 1 Phil. Trans. Roy. Soc. London, 203B, p. 1
Syrbula	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Syrbula acuticornis	20 spg	10♂	10♂	10♂	Bivalent heterochrom, divides in both divisions	Montgomery, '05	Proc. Acad. Nat. Sci. Phila., 57, p. 162
Syrbula admirabilis	23 spg	12♂	11, 12♂	11, 12♂	X to pole in 1st	Robertson, '08	Kansas Univ. Sci. Bull., 4, p. 275
Tettigidae (subfamily of Acrididae)							
Acridium granulatus. Paratettix Tettigidea parvipennis	13 spg 14 spg	7♂			X to pole in 1st	Robertson, '08 Robertson, '15	Kansas Univ. Sci. Bull., 4, p. 275 Jour. Morph., 26, p. 109
Paratettix [illegible] leucothorax	13 spg	7♂	6, 7♂		X to pole in 1st	Harman, '15	Biol. Bull., 24, p. 262
Trimerotropis	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Tropidolophus	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651
Tryxalis	23 spg	12♂	11, 12♂		X to pole in 1st	McClung, '14	Jour. Morph., 25, p. 651

Tryxalis nasuta..........	21 spg	11♂			X to pole in 1st	Brunelli, '10 Brunelli, '11	Mem. Soc. ital. Sci., ser. 3a, vol. 16, p. 221 Mem. R. Acad. Lincei, ser. Va, vol. 8, p. 633
			b. Blattidae				
Blatta germanica.........		12♂				Erlanger, '96 Erlanger, '97	Zool. Anz., 19, p. 65 Zool. Centralb., 4, p. 1
Blatta germanica.........	23 spg 24 oog	12♂	11, 12♂	11, 12♂	X to pole in 1st	Wassilieff, '07	Arch. mikr. Anat., 70, p. 1
Blatella germanica (= Blatta)...............	23 spg 23? ♀ som	12♂	11, 12♂		X to pole in 1st	Stevens, '05	Carnegie Inst. Pub. no. 36, p. 1
Leucophaea maderiae.....	23 spg 24 oog		11, 12♂		X to pole in 1st	Morse, '09	Arch. Zellf., 3, p. 483
Periplaneta americana....	32 spg	16♂ (gemini)	16♂			Farmer and Moore, '04 Moore and Robinson, '04 Moore and Arnold, '05	Q. J. M. S., 48, p. 489 Q. J. M. S., 48, p. 571 Proc. Roy. Soc., 77, p. 563
Periplaneta americana....	33 spg 34 oog	17♂	16, 17♂	16, 17♂	X to pole in 1st	Morse, '09	Arch. Zellf., 3, p. 483
			c. Forficulidae				
Anisolabis maritima......	24 spg 24 oog 24 som	12♂	12♂	12♂	Equal pair heterochromosomes	Randolf, '08	Biol. Bull., 15, p. 111
Forficula auricularia......		10–14♂				Carnoy, '85	La Cellule, 1, p. 189
Forficula auricularia......		12♂	12–14♂			St. George, '87	Festschrift. f. Kölliker, p. 51
Forficula auricularia......	24 spg	12♂	12♂			Sinéty, '01	La Cellule, 19, p. 117
Forficula auricularia......	24 or 26 spg	12–14♂	12–14♂	12–14♂	One accessory chrom. (13) or two (14) or none (12)	Zweiger, '06 Zweiger, '06	Zool. Anz., 30, p. 220 Jen. Zeits., 35, p. 143
Forficula auricularia......	24 spg	12♂	12♂ (occasionally 11 and 13)	12♂	XY to poles in 1st	Stevens, '10	Jour. Exp. Zool., 8, p. 227

II. ARTHROPODA—Continued

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Forficula auricularia......	24 spg	12♂	12♂		Pair heterochromosomes in 2nd	Meck, '13 Meck, '13 Meck, '15	Phil. Trans. Roy. Soc., London, 203B, p. 1 Q. J. M. S., 59, p. 249 Q. J. M. S., 61, p. 1
Forficula auricularia(?)...	24-27 spg	12-14♂	11-14♂	10-14♂	Some chroms. univalent in 1st and may or may not divide	Payne, '14	Jour. Morph., 25, p. 559
Labidura riparia...........		6♂				Sinéty, '01	La Cellule, 19, p. 117
			d. Gryllidae				
Gryllotalpa...............	23 spg	12♂				Baumgartner, '12	Science, 35, p. 937. (Paper read before Amer. Soc. Zool.)
Gryllotalpa borealis......	23 spg 24 oog	12♂	11, 12♂		X and XY to poles in 1st	Payne' 12	Arch. Zellf., 9, p. 141
Gryllotalpa vulgaris	12 spg	6♂ = 24 el.	6♂ = 12 el.	6♂	Tetrads and dyads = separate elements	Vom Rath, '92 Vom Rath, '95	Arch. mikr. Anat., 40, p. 102 Arch. mikr. Anat., 46, p. 168
Gryllotalpa vulgaris......	17 spg	9♂			X to pole in 1st	Senna, '11	Mon. Zool. ital., 22, p. 65
Gryllotalpa vulgaris......	16 spg (= 17, one bivalent)	7♂	7♂	7♂	XY to poles in 1st. X united with a tetrad. X to pole in 1st. Two tetrads united in 1st. From correction in '14	Voinov, '12 Voinov, '14	C. R. Assoc. Biol., 72, p. 621 Arch. Zool. Exper. et gen., 54, p. 439
Gryllus assimilis (luctuosus?)..................	29 spg	15♂	14, 15♂		X to pole in 1st	Baumgartner, '04	Biol. Bull., 8, p. 1
Gryllus desertus..........	21 spg	11♂			X to pole in 1st	Brunelli, '09	Mem. R. Acad. Lincei., ser. 5a., vol 7, p. 623
Gryllus domesticus	21 spg	11♂	10, 11♂	10, 11♂	X to pole in 1st	Baumgartner, '04	Biol. Bull., 8, p. 1

Gryllus domesticus.......	21 spg 22 oog 21♂ som 22♀ som	11♂			X in ♂; XX in ♀	Gutherz, '07	Arch. Mikr. Anat., 69, p. 491
						Gutherz, '08	Zentr. Physiol., 22, p. 61
						Gutherz, '09	Sitz. Gesel. Naturf. Fr. Berlin, 1909, pp. 410 and 565
Gryllus domesticus	21 spg	11♂	10, 11♂		X to pole in 1st	Meek, '13	Phil. Trans. Roy. Soc., London, 203B, p. 1
			c. Locustidae				
Anabrus	33 spg	17♂	16, 17♂	16, 17♂	X to pole in 1st	McClung, '02	Kansas Univ. Sci. Bull., 1, p. 185
						McClung, '14	Jour. Morph., 25, p. 651
Ceuthophilus sp?.........	37? spg	19♂ or 21♂ 2S)			X to pole in 1st. 2S to either pole in 1st or 2nd	Stevens, '12	Biol. Bull., 22, p. 219
Conocephalus.............	33 spg					McClung, '14	Jour. Morph., 25, p. 651
Decticus verrucivorus....	23 spg	12♂	11, 12♂	11, 12♂	X to pole in 1st	Vejdovsky, '12	Kgl. Bohm. Gesel. Wissen. Prag, 1912, p. 1
Decticus verrucosus......	31 spg					Buchner, '09	Arch. Zellf., 3, p. 335
Diestrammena marmorata.....................	57 spg	28♂	28, 29♂		X to pole in 1st	Schellenberg, '13	Arch. Zellf., 11, p. 489
Jamaicana flava Jamaicana subguttata Jamaicana unicolor	35 spg	18♂	17, 18♂	17, 18♂	X to pole in 1st Association of 2 chroms. in some individuals	Woolsey, '15	Biol. Bull., 28, p. 163
Leptophyes punctatissima....................	31 spg 32 oog 31♂ som 32♀ som			15, 16♂	X to pole in 1st. XX in ♀ diploid	Mohr, '15	Arch. Zellf., 14, p. 151
Locusta viridissima	33 spg	17♂	16, 17♂	16, 17♂	X to pole in 1st	Otte, '06	Zool. Anz., 30, p. 529
						Otte, '07	Zool. Jahrb., 24, p. 431
Locusta viridissima.......	29 spg 30 oog 29♂ som 30♀ som				X to pole in 1st. XX in ♀ diploid	Mohr, '15	Arch. Zellf., 14, p. 151

II. ARTHROPODA—Continued

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Microcentrum	33? spg	17♂	16, 17♂	16, 17♂	X to pole in 1st	McClung, '02	Kansas Univ. Sci. Bull., 1, p. 185
Orchesticus	33 spg	17♂	16, 17♂	16, 17♂	X to pole in 1st	McClung, '02	Kansas Univ. Sci. Bull., 1, p. 185
						McClung, '14	Jour. Morph., 25, p. 651
Orphania denticauda	31 spg	15♂ (= 16, X at pole in metaphase)	15, 16♂	15, 16♂	X to pole in 1st	Sinéty, '01	La Cellule, 19, p. 117
Scudderia	33 spg	17♂	16, 17♂	16, 17♂	X to pole in 1st	McClung, '02	Kansas Univ. Sci. Bull., 1, p. 185
						McClung, '14	Jour. Morph., 25, p. 651
Steiroxys trilineata	29 spg	15♂	14, 15♂	14, 15♂	X to pole in 1st	Davis, '08	Bull. Mus. Comp. Zool. Harvard, 53, p. 57
Steiroxys trilineata	29 spg	15♂	14, 15♂		X to pole in 1st	Meek, '13	Phil. Trans. Roy. Soc. London, 203B, p. 1
Stenopelmatus	47 spg		23, 24♂		X to pole in 1st. (From correction in '09)	Stevens, '05	Carnegie Inst. Pub. no. 36, p. 1
						Stevens, '09	Jour. Exp. Zool., 6, p. 101
Troglophilus sp?	ca. 20 oog					Buchner, '10	Arch. Zellf., 5, p. 449
Xiphidium	33 spg	17♂	16, 17♂	16, 17♂	X to pole in 1st	McClung, '02	Kansas Univ. Sci. Bull., 1, p. 185
						McClung, '08	Kansas Univ. Sci. Bull., 4, p. 255
						McClung, '14	Jour. Morph., 25, p. 651
f. Mantidae							
Mantis religiosa	14 spg	14♂ (double = 28)	7♂ (double = 14) 14♀	7♂, 14♂ pron 7♀, 14♀ pron	Chroms divide just before fertilization	Giardina, '97	Mon. Zool. ital., 8, p. 275

g. Phasmidae

Aplopus mayeri..........	35 spg 36♀ som	18♂	17, 18♂	17, 18♂	X to pole in 1st	Jordan, '08 Jordan, '08	Anat. Anz., 32, p. 284 Carnegie Inst. Pub. 102, p. 13
Bacillus linearis..........		8-10♂				Carnoy, '85	La Cellule, 1, p. 189
Bacillus rossii............	20 oog 20 cl	18-20♀	ca. 20♀		Parthenogenetic. 2 polar bodies	von Baehr, '07 von Baehr, '12	Zool. Jahrb., 24, p. 175 La Cellule, 27, p. 383
Leptynia attenuata.......	36 spg (= 37) 36 oog	18♂ (= 19)	18♂ (= 18, 19)		X attached to another chrom. To pole in 1st	Sinéty, '01	La Cellule, 19, p. 117

d. MYRIAPODA

1. *Chilopoda*

Geophilus linearis........		8♂	8♂	8♂		Bouin and Collin, '01 P. and M. Bouin, '03	Anat. Anz., 20, p. 97 C. R. Soc. Biol., 55, p. p. 763
Lithobius forficatus		16-24♂				Carnoy, '85	La Cellule, 1, p. 189
Lithobius forficatus.......		22-24♂				P. and M. Bouin, '02	C. R. Assoc. Anat., 4, p. 74
Lithobius mordax Lithobius multidentatus Lithobius sp?		25♂			X	Blackman, '07	Proc. Amer. Acad. Arts and Sci., 42, p. 487
Scolopendra dalmatica		20-24♂				Carnoy, '85	La Cellule, 1, p. 189
Scolopendra heros........	33 spg	17♂	16, 17♂	16, 17♂	X to pole in 1st	Blackman, '03 Blackman, '05	Biol. Bull., 5, p. 187 Bull. Mus. Comp. Zool. Harvard, 48, p. 1
						Blackman, '10	Biol. Bull., 19, p. 138
Scutigera coleoptrata.....		18♂			Accessory divides in both divisions	Bouin and Ancel, '11	C. R. Assoc. Anat., 13, p. 104
Scutigera forceps.........	37 spg	19♂	18, 19♂		X to pole in 1st	Medes, '05	Biol. Bull., 9, p. 156

2. *Diplopoda*

Pachyiulus varius........	25 spg	13♂	12, 13♂		X to pole in 1st	Oettinger, '08 Oettinger, '09	Zool. Anz., 33, p. 164 Arch. Zellf., 3, p. 563
Polyxenus sp?............	prob. 16 spg	8♂	8♂			Sokoloff, '14	Zool. Anz., 44, p. 558

II. ARTHROPODA—Continued

e. Onychophora

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Peripatus .	28 spg (23–34) 28 oog 28 som	14 ♂	14 ♂	14 ♂		Montgomery, '00	Zool. Jahrb., 11, p. 277

III. COELENTERATA

a. Hydrozoa

1. Leptolinae

a Anthomedusae

SPECIES	DIPLOID AND PARTHENOGENETIC	1ST -CYTE	2ND -CYTE	-TID	REMARKS	OBSERVER	REFERENCE
Clava squamata..........		ca. 16 ♀				Harm, '02	Zeit. wiss. Zool., 73, p. 115
Clavatella prolifera (= Eleutheria dichotoma)		6 ♀				Müller, '08	Zeit. wiss. Zool., 89, p. 28
Cordylophora lacustris...		10–12 ♀				Morgenstein, '01	Zeit. wiss. Zool., 70, p. 567
Eudendrium ramosum...		13? ♀				Beckwith, '14	Jour. Morph., 25, p. 189
Hydra dioecia Hydra fusca Hydra viridis	12 spg	6 ♂ 12 ♀	6 ♂ 6 ♀	6 ♂ 6 ♀	Reduction in 1st anaphase in ♀	Downing, '05 Downing, '09	Zool. Jahrb., 21, p. 379 Zool. Jahrb., 28, p. 295
Hydra grisea............		12–14 ♀				Brauer, '91	Zeit. wiss. Zool., 52, p. 169
Hydra grisea .		16 ♀				Wager, '04	Biol. Bull., 18, p. 1
Hydractinia echinata.....	12–16 cl					Smallwood, '09	Biol. Bull., 17, p. 209
Hydractinia echinata.....		14? ♀				Beckwith, '14	Jour. Morph. 25, p. 189
Pennaria tiarella		10-14 ♀ (prob. 10)				Hargitt, '09	Bull. Mus. Comp. Zool. Harvard, 53, p. 161
Pennaria tiarella..........	14? cl					Smallwood, '09	Biol. Bull., 17, p. 209

Tiara sp?	28 cl	14 ♀	14 ♀	14 ♀		Boveri, '90	Zellenstudien III, or Jen. Zeits., 17, p. 314
Tubularia mesembryanthemum		ca. 12 ♀				Brauer, '91	Zeit. wiss. Zool., 52, p. 551
			b. Leptomedusae				
Aequorea forskalea	12 cl		6 ♀			Häcker, '92	Arch. mikr. Anat., 40, 243
Campanularia fluxuosa	20 cl	10 ♀				Hargitt, '13	Jour. Morph., 24, p. 383
Gonothyrea loveni		8 ♀				Wulfert, '02	Zeit. wiss. Zool., 71, p. 296
			2. Trachylinae				
Cunina proboscidea		28+♂ 30 ♀		14+♂		Stschelkanowzew, '06	Mit. Zool. St. Neapel, 17, p. 433
Gonionemus murbachii	24-25 spg prob. 24 oog 24 cl prob. 24 som	prob. 12♂	prob. 12♂	prob. 12 ♀		Bigelow, '07	Bull. Mus. Comp. Zool. Harvard, 48, p. 287
			b. SCYPHOZOA				
Aurelia flavidula	18-20 cl	9-10 ♀				Hargitt, '10	Jour. Morph., 21, p. 217

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A COMPARATIVE STUDY OF THE CHROMOSOMES OF SIX SPECIES OF NOTONECTA[1]

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ONE HUNDRED AND FIFTEEN FIGURES (SEVEN PLATES)

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I. INTRODUCTION

A previous study of the chromosomes of three species of Notonecta (Browne '13) proved of such interest as to lead me to undertake a more comprehensive study of the chromosomes of this genus. It is with the view of making a comparative cytological examination of all the species of this genus that the present paper is written dealing with three additional species. Owing to the fact that certain chromosomes are characteristic in size, position and behavior, and also to the fact that the differences in the chromosome number and grouping are relatively slight in the different species, Notonecta offers an exceptionally good field for such a comparative study. In order to ascertain the relation between the chromosomes and somatic characters, one of the chief aims of modern cytology, a comparative cytological investigation of closely related forms seems a method of attack which should accompany the experimental method. Moreover, it would seem that the study of different chromosome groups within a genus should give a clue to the relationship and possible evolution of the different species, so that a phylogenetic tree based on the chromosomes would correspond with one based on somatic characters and geographical distribution. A thorough cytological examination has now been made, at least with regard to the spermatogenesis, of six out of twenty species of Notonecta. This comparatively limited study will, of course, not guarantee any sweeping or final statements as to the relation of particular chromosomes to particular body characters, or as to the precise phylogeny of the species; only a few general and tentative suggestions in this regard are offered in this paper.

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[1] This work was done while holding the Sarah Berliner Research Fellowship.

The problem concerning the chromosomes as giving a clue to the relationship of different forms is one to which Montgomery ('01, '06) devoted considerable attention. He argued that the chromosomes are conservative elements and less modifiable than the soma, and that they should therefore be considered as of value in determining the relationship of a particular group. He was, however, dealing with the Hemiptera in general and was considering only the numerical relations of the chromosomes. It is not surprising, therefore, that he found this problem increasingly difficult of solution as his study became more extensive. A glance at the chromosome numbers of different species of Hemiptera as listed by him in 1906 will show how difficult such a task would be. Had he limited himself to families, an effort to find a rational explanation of a diversity in number of chromosomes corresponding with the relationship of the genera, would in many cases probably have proved fruitless. It has been repeatedly shown by the work of Wilson, Boring, Stevens, Morgan and many others that even within certain genera, there is a discrepancy in the number of chromosomes in the different species entirely disproportional to the differences in body characters; for example, different species of phylloxerans have 6, 8, 12, and 22 chromosomes (Morgan '09). So true is this in the case of certain Homoptera, that Boring ('07), after a study of twenty-two species, concluded that the number of chromosomes is of no significance for grouping species into genera. On the other hand, there are fortunately other groups which are quite favorable for a solution of this problem. The orthopteran family, Acrididae, has engaged the attention of McClung for some time in an effort to discover the relation between chromosomes and somatic characters. The forty genera already examined, have, with a few exceptions, shown the same number of chromosomes, and the constancy in number, size and form of the chromosomes has led McClung ('05, '08, '14) to a belief in their importance in taxonomy. A peculiar association of some of the chromosomes in certain genera and species is believed by him to be directly responsible for the generic or specific somatic characters of those forms. The genus Notonecta, consisting as it does of many species differing from each other slightly, but characteristically, both in somatic characters and in chromosome content, offers another very favorable object for the study of this problem.

II. SPECIES OF NOTONECTA

The genus Notonecta includes, according to the entomologists, Kirkaldy ('97) and Bueno ('05), twenty species, thirteen of which are peculiar to America. One species (N. lutea) is common to America, Europe and Asia; one (N. glauca) is common to Europe, Asia and Africa; one (N. lactitans) is African; one (N. handlirschli) is Australian; and three (N. chinensis, N. montandoni, N. triguttata) are Asiatic. My former paper (Browne '13) dealt with three of the American forms common in the eastern United States, N. undulata, N. irrorata, and N. insulata. The present paper deals with the European N. glauca, and two species common in the western United States, N. shooterii and N. indica. The other American species are N. variabilis, N. uhleri, N. howardii, N. raleighi, N. mexicana, N. montezuma, N. bifasciata and N. nigra, the two latter ones being peculiar to South America. The genus Notonecta, together with Buenoa and the Pleinae belong to the family Notonectidae.

III. BRIEF SUMMARY OF THE CHROMOSOMES OF N. UNDULATA, N. IRRORATA, AND N. INSULATA

For a full and detailed account of the chromosomes of these species, the reader is referred to my former paper (Browne '13). I here merely outline the chief features of the chromosome groups in order to have a basis for reference and comparison with those of the three other species described in detail in this paper.

In N. undulata the number of chromosomes in the first spermatocyte division is 14, consisting of a ring of 12 chromosomes surrounding two small central ones (fig. 1). In side views, these central pairs often appear linearly arranged, owing to their proximity and to the precocious division of one pair (fig. 2). In the second division the number is reduced by one because of the late conjugation of X and Y, which are not associated in the first division. In this division the XY dyad lies in the center of a ring of 12 chromosomes (figs. 3 and 4). The components of this pair are considerably unequal in size, and, owing to their presence, the sister groups which go into the spermatids and spermatozoa are of two kinds (fig. 5 A, B). The spermatogonial group contains 26 chromosomes (fig. 6). There are five large chromosomes in the diploid group, representing two large ones of the haploid and X which is next in size to these. There are five small chromosomes, representing the two small central ones of the first division and Y which is the next smallest after these.

In N. irrorata, the number of chromosomes in the first division is 13, and there is only one small one within the ring (figs. 7 and 8). In the second division there are 12 chromosomes and the XY dyad lies in the center of the ring (figs. 9 to 11). The spermatogonial number is 24 (fig. 12). There are seven large chromosomes, corresponding to three large ones of the haploid group and X which is next in size. There are six small chromosomes corresponding to the one in the center in the first division, and two small peripheral ones; Y is one of the next smallest.

In N. insulata there are two types of first division groups, one with 14 chromosomes including two small ones in the center, like N. indulata, and the other type with 13 chromosomes including only one small one in the center, like N. irrorata (figs. 13 to 16). When there is only one small one in the center, the other one is often found attached to the largest chromosome (fig. 16). There are 12 chromosomes in the second division including the XY dyad lying in the center (figs. 17 to 19). It is assumed that the second small chromosome is always fused with the largest one in this division. No diploid groups have been found in which the number could be determined. In the haploid groups, a largest and usually a next largest chromosome are noticeable and another small one in addition to the one (or two) in the center in the first division.

IV. THE CHROMOSOMES OF N. GLAUCA

It has been with particular interest that I have examined the chromosomes of N. glauca because of the previous work of Pantel and Sinéty ('06) on the same species. Their account of the maturation divisions is so very meager that I feel justified in giving my own observations in full. I wish to express my sincere thanks to Professor Lefroy of The Royal College of Science, London, for his kindness in supplying me with the insects last summer, and to Professor Dobell, in whose laboratory the material was prepared.

a. Observations

In N. glauca there are, in the first division, 13 chromosomes, which appear in polar view as a ring of 12 surrounding one small central one (figs. 20 to 22). The most striking feature of this group, apart from the general arrangement, is the presence of an exceptionally large chromosome which appears as a double or compound chromosome. A similar appearance is sometimes presented by the next largest chromosome (fig. 20), though in the majority of cells this is not the case (figs. 21 and 22). A longitudinal section through the center of the spindle shows the small dyad on the central fibers and the large compound chromosome and the next largest one on the peripheral fibers (fig. 23). Consecutive sections of a spindle show the appearance of the entire group of 13 in side view, including the small central dyad, the large compound chromosome, the next largest, somewhat similar in appearance, and another small one, a trifle larger than the central one (fig. 24 A, B, C). Very frequently, the components of the small central dyad lie on different fibers and are not linearly arranged (fig. 25 B). This has been noted previously in N. irrorata, and likewise occurs in N. shooterii. This peculiarity is probably due to the fact that the components of this dyad do not become associated in the late prophase as the components of the other chromosomes do; even when linearly arranged, the components of this small chromosome are usually considerably separated in the full metaphase. Such a precocious separation of the parts of the small central chromosome (the m-chromosome) seems to be characteristic also of the coreids (Wilson et al.). That the small chromosomes in the center in figure 25 B are the components of one central dyad, and do not represent two small dyads is shown by the fact that these chromosomes are distinctly univalent in contradistinction to the other bivalents, and they are passing to opposite poles; moreover, the complete number of 13 is given, only if these are regarded as one chromosome (fig. 25 A, B). Particular attention has been paid to this question, for if this interpretation be not correct, the explanation that the difference between the 14 chromosome groups of some species and the 13 groups of other species is due to the presence or absence of the second small chromosome in the center, would not stand. However, I am convinced from a very careful study of many spindles in side and polar view, that there can be no question that these are the components of one small central dyad and are not two separate dyads.

The large compound chromosome presents a variety of appearances as seen in side view (figs. 23, 24 C, 25 A and 26). In general it may be described as consisting of four open U's, with the arms often pulled out transversely, sometimes giving it the appearance of being combined with a smaller chromosome. The darker portions in the drawings represent dark areas seen on the chromosome, probably due to the overlapping of two parts, causing thickenings in these regions. Frequently, when the chromosome is seen in face view, that is, on the top of the spindle instead of at the side, a characteristic double cross is apparent (fig. 27). In polar view, the usual appearance is of two diverging, slightly curved arms or open U's (figs. 20 to 22); this probably represents only half of the entire chromosome, for often a more complex structure is evident, consisting of a system of superimposed open U's (fig. 28). In early anaphase, the chromosome appears somewhat irregular (figs. 29 and 30), but the structure of each sister half is clear in later anaphase (figs. 31 and 32). Each part consists of two slightly curved arms or open U's in contact at the base, and bending away from each other. The peculiarity and irregularity of the chromosome in metaphase is probably due to a difference in the amount of fusion and the arrangement of its component parts. The structure of the next largest chromosome, which I described as sometimes similar in appearance to the largest chromosome in the metaphase, is in the anaphase similar in structure, only the parts are smaller and not quite so curved (figs. 31 and 32). These chromosomes in polar views of the anaphase give approximately the same appearance as in side view (fig. 33); this figure shows the entire group of chromosomes in polar view, similar in grouping and size relations to the polar metaphase. Occasionally the chromatic substance from the large chromosome seems to run down over the spindle fibers in the anaphase, giving the figure a very curious appearance (fig. 34). Other observers have noted a thickening of the spindle fibers where attached to the chromosomes, as though some of the chromatic substance contributed to them. This appearance is usual in Notonecta, as I have represented in the drawings, and it is probably an extreme exaggeration of this tendency that is represented in figure 34.

In the telophase of the first division, which is also the prophase of the second, the chromosomes become somewhat crowded, but it is often possible to distinguish the individual ones. The large compound chromosome retains its structure of the anaphase, and can in most cases be followed with ease at this time, and this is also true of the next largest one (fig. 35); both these chromosomes, probably on account of their bulk, usually lag behind the others in division. When on the second spindle (fig. 36), the line of separation between the two curved bars falls along the equatorial plane. In the case of the largest chromosome, therefore, the line of separation in the second division is already marked out in the metaphase of the first, and is in evidence in all the following stages. A side view of the second division shows a characteristic XY pair in the center of the spindle (figs. 36 and 37 B). Owing to the fact that X and Y divide as separate chromosomes in the first division, and as a single chromosome in the second, the number is reduced by one. Consequently, in serial sections of a complete spindle, there are twelve chromosomes, including the XY dyad in the center, and on the peripheral fibers the large compound chromosome, the next largest similar in appearance, and the two small chromosomes, one of which was in the center in the first division (fig. 37 A, B, C). Frequently, the X and Y chromosomes do not take up a linear arrangement, but lie side by side in the metaphase, on different spindle fibers, a peculiarity found in all the species of Notonecta and comparable with the non-linear arrangement of the components of the small central dyad in the first division; this arrangement seems to occur only in those chromosomes whose components normally conjugate late. An entire spindle in which this arrangement of X and Y occurs, is shown in figure 38 A, B, C. Polar views show the same arrangement and size relations of the chromosomes as side views, and sometimes X and Y lie side by side at the same focus (figs. 39 and 40). Whether the two large chromosomes appear double (fig. 39) or single (fig. 40), depends of course, upon whether both sister elements are in view or only one of them. The difference in the amount of chromatin contained in the large chromosomes and the small ones is well shown in figure 41, where these four chromosomes happen to lie together.

The inequality in the X and Y chromosomes is quite striking in most cases in N. glauca, but is not so great as in the species previously described. There seems to be some individual variation in this respect, the XY pairs from one individual (fig. 42) being more nearly equal than in another (fig. 43). This variation is more marked in N. shooterii, in connection with which the question is discussed more fully. I have found no case in N. glauca in which I have been unable to detect an inequality. This species resembles Nezara hilaris (Wilson '11) in having a constant but often slight difference in the components of this pair, although this difference is perhaps greater in N. glauca. In anaphase, X and Y pass to opposite poles and also the sister elements of the two large chromosomes which at this time are less characteristic in shape (fig. 44). In the late anaphase (figs. 45 and 46) these chromosomes are noticeable as long, somewhat curved bars, and sometimes the larger one gives the appearance of being double on account of a notch in the center, a remnant possibly of the bend in the original U which was one of the four parts composing the large chromosome. Sister anaphase groups in polar view are practically identical except for the central chromosomes which differ in size, sometimes considerably (fig. 47), sometimes less noticeably (fig. 48). Owing to this fact of course, the spermatids and likewise the spermatozoa will be of two kinds with regard to their chromatin content, bearing the usual relation to sex-production.

The growth of the cells in N. glauca is very slow, as has been noted also by Pantel and Sinéty, and on this account most of the cysts containing spermatogonia and early growth stages are empty at the time that the division stages occur, so that few dividing spermatogonia are to be found in the material used for the maturation divisions. The best one occurring in my material is shown in figure 49, and although the number cannot be counted with certainty, the group shows the structure of the two largest chromosomes which appear as long curved rods. This figure also shows the presence of four small chromosomes corresponding with the central one and the small peripheral one of the first division. Female diploid groups show 24 chromosomes, including four small ones and six large ones, the third pair of large ones being in all probability the X chromosomes (fig. 50).

b. Comparison with the results of Pantel and Sinéty

Pantel and Sinéty, in their monograph on "Les cellules de la lignée mâle chez le Notonecta glauca," have been concerned chiefly with the growth stages and transformation into the spermatozoon, and have only incidently taken up the maturation divisions. However, their criticism of the chromatic figure as being "d'un type malingre" for the study of these divisions seems to me to be altogether unwarranted, for the chromosomes are large, well differentiated in size, and in general seem exceptionally fine for study. Judging from the few figures that are given of these stages, their material was essentially like my own and was prepared in the same way, and I believe that with further study would have yielded similar results.

In the first division, they count in polar views, sometimes 12, sometimes 13 chromosomes, arranged in a ring with one or two in the center, but they state that they can assign no reason for the discrepancy in number. However, in a part of their paper dealing with abnormalities—here the presence of small deep staining corpuscles—a polar view is given, showing a group of 12 chromosomes surrounding one small one, the grouping and size relations corresponding exactly with my figures. The large chromosome, which is such a striking feature of this species, has received particular attention from these authors. They call it the 'chromosome exceptionelle' and describe it as a massive irregular body giving one the impression of a double cross or a system of double V's, and their figures of it in polar metaphase and side anaphase are quite similar to mine. Although they are convinced that it participates in both divisions, they suggest a possible relationship to an accessory chromosome; they abstain however from giving its exact significance. In the second division, they do not state the number of chromosomes, but a figure of a polar view shows 12, including one inside the ring, and the 'chromosome exceptionelle' having a cross shape, the next largest of a similar shape, and two small ones, a group similar to those that I have figured. These authors have apparently overlooked entirely the XY chromosomes, and this may explain their hesitancy in stating the number of chromosomes in the two divisions, which should differ by one with these chromosomes present, and should be the same if they are absent.

V. THE CHROMOSOMES OF N. SHOOTERII

A study of the chromosomes of N. shooterii has shown that the number is the same as in N. undulata, but that the grouping is more like that of N. irrorata and N. glauca.

a. Observations

The complete number of chromosomes in N. shooterii in the first spermatocyte division is 14, consisting of one small central one surrounded by a ring of 13 (fig. 51). It is characteristic of this species that a second small chromosome lies usually just within the peripheral ring, but sometimes a little more toward the center (fig. 52). It does not lie typically close to the central one, as is characteristic of N. undulata. The position of these two small chromosomes is more strikingly seen in side view, one of them appearing on the central fibers, and the other one some little distance away on the peripheral fibers (figs. 53 and 54 B). In the actual sections, the position of the second small chromosome somewhat within the peripheral ring is perfectly evident from the fact that it lies at a different focus from the others in the periphery; this cannot be so well shown in the drawings, although I have attempted to do so by different shading. A somewhat oblique section perhaps brings this out more clearly (fig. 55). It often happens in N. shooterii, as described also for N. irrorata and N. glauca, that the components of the central dyad lie side by side, instead of being linearly arranged (fig. 56). When such a spindle is followed through in consecutive sections, the other 13 chromosomes are to be found, including the second small one, just within the peripheral ring.

In the anaphases, the components of the small central chromosome always lead in the procession toward the poles. The second small chromosome from within the peripheral ring is to be found directly behind the smallest one (figs. 57 B to 61). It is obvious that the mechanical arrangement more or less necessitates the approach of these chromosomes to the pole before the others that are on the outer fibers, and this may be the only explanation of this fact. However, in many cases, these two small chromosomes seem to be connected by fibers, but it is impossible to be sure of this owing to the delicacy of the structures concerned. In a polar view of the late anaphase, the two smallest chromosomes are often to be found quite close together within the ring of 12 other chromosomes (fig. 61). From the position and behavior of the second small chromosome, I believe that it is homologous with the second small one in the center in N. undulata.

In the second division, a characteristic X Y pair is seen in the center of the spindle, surrounded by a group of 12 chromosomes arranged on the peripheral fibers (fig. 62 A, B, C). Among these may be seen the two small chromosomes of the first division. A polar view shows a ring of 12 chromosomes, including two small ones, surrounding X or Y (figs. 63 and 64). These chromosomes are frequently found side by side in the metaphase as described for N. glauca (fig. 65 A). Figure 67 A, B, shows a polar view of two sister groups of chromosomes which have just separated, these being taken from a rather thick section (7μ), where they were at different levels.

The size relations of X and Y are quite variable, as will be noticed by a glance at the figures. In some cases these chromosomes are almost equal, in others they are quite unequal (figs. 62 to 74). The sister groups, therefore, of the late anaphase, are sometimes quite dissimilar in their chromatin content, with respect to the central chromosome, and sometimes almost identical (figs. 75 to 77). In the spermatogonial groups, there are 26 chromosomes, including five large ones, four of which correspond with the two largest ones of the spermatocytes, and the fifth being X; there are four small ones corresponding with the small central one and the one just within the ring in the first division (figs. 78 and 79). Although X and Y are sometimes almost equal in the haploid groups, I have found no case of equality in the diploid, even from the same individual. The oogonial groups give the same number of chromosomes, 26, but have six large ones instead of five, the sixth large one being the second X chromosome (fig. 80).

b. Size relations of X and Y

The X and Y chromosomes, as was mentioned above, differ considerably in their relative sizes in different cells, and this is true to a certain extent for different individuals. Although in many individuals, the inequality of X and Y is considerable, in others the inequality is less marked, and in some cells of these individuals is not apparent at all. This relative inequality is, however, not constant, for in those individuals where there is a tendency to equality, certain cells are found where the inequality is marked. In figures 68 to 74, there are shown the X Y pairs from seven different individuals, the drawings being made as carefully as possible and the extremes and means of inequality being represented in each case. The inequality is well marked in figure 68, fairly evident in figures 69 and 70, less so in the others. In figures 73 and 74, X and Y are in some instances apparently equal, though in one case in each individual, they are markedly unequal. A variation in the size of these chromosomes has been found also by Wilson ('12), in Oncopeltus, and the percentage of cases of equality and inequality was found to vary widely in different individuals in this instance also.

The variation in the relative size of X and Y is well shown in sister polar views of the anaphase (figs. 75 to 77). Since these groups go directly into the spermatids and spermatozoa, it is evident that in certain cases (where X and Y are different in size), these will fall into two classes, differing in their chromatin content. In those cases, however, where X and Y are equal (fig. 77), there will be no visible difference. Such groups as these lead, of course, to those forms, of which there are probably many, where there is never a visible dimorphism in the chromatin content of the spermatids; and yet there is reason to believe that such a dimorphism exists. This important fact has been pointed out by Wilson in considering Oncopeltus, and additional evidence is given by the same phenomenon in N. shooterii. There is absolutely no question in this case of the existence of an X Y pair, as these chromosomes are typical and constant in position and behavior in all the species of the genus thus far examined.

VI. THE CHROMOSOMES OF N. INDICA

N. indica has the same number of chromosomes as N. undulata and N. shooterii, and they are arranged in the first division somewhat like those of N. undulata, but slightly modified.

a. Observations

In N. indica, there are two small chromosomes in the center, as shown by spindles in side view (fig. 81). Owing to the proximity of these two chromosomes and to the fact that one pair divides in advance of the other, they most frequently appear arranged in a linear series (fig. 82). This appearance is common in N. undulata also. Three other very characteristic chromosomes appear in side view, two of these being large compound chromosomes, similar to the largest chromosome of N. glauca, only smaller, and a third chromosome, largest of all which consists of a main body and usually a small piece on either side, looking like a compound chromosome, made up of one large and two very small parts. This chromosome can be traced through from early prophase of the first division to the telophase of the second by its characteristic shape, and is the larger component X of the X Y pair. The double chromosomes and the X chromosome are shown as they lie on the spindle in figures 83 and 84. A complete set of chromosomes as seen in side view gives a total of 14, including X, the two doubles and the two small ones in the center (fig. 85 A, B, C). Polar views showing the complete number 14, the general grouping and the size relations are given in figures 86 to 88. The arrangement seems somewhat irregular at first sight, but closer examination shows a more or less regular ring of ten chromosomes, with the two smallest ones in the center and two intermediate ones between these and the periphery. This arrangement seems quite constant, but owing to the fact that it is somewhat complex, I can not be sure that it is invariable. A slight shifting of one of the chromosomes from the periphery would serve to confuse the arrangement. In side views, the position of these two ex-centric chromosomes is a little diffcult to determine, except when they happen to lie in the section which includes the two small chromosomes and in a plane determined by the central and outermost chromosomes, as indicated by focusing. The ex-centric chromosomes determined in this way are indicated in light gray in figures 81, 82 and 85 B. It appears that these are not the smallest chromosomes after the central one, but are among the intermediate ones. In the polar views, the large double chromosomes appear almost like crosses; in many cases it is impossible to tell whether the division line between the two curved bars or U's is in line with the diameter of the circle or at right angles to it. In fact the chromosome is sometimes turned so that the line of separation lies midway between these two positions (fig. 87). In the anaphase, a peculiar shape is often assumed by X (fig. 89), but after the two sister elements are separated, each shows again the appearance of being composed of a larger and smaller parts (figs. 90 and 91). In these figures are also shown the sister elements of the large compound chromosome (in figure 91, both large ones), each consisting of two curved bars or open U's, bent away from each other. In figure 92, A, B, C, are given serial sections of a spindle in anaphase, showing all the 14 chromosomes in each sister group.

The appearance of X[2] in the early prophase of the first division is even more remarkable than in later stages. Its main central mass is split longitudinally, and at each end of each bar are small knobs attached to the main mass by thin threads. The number of these smaller bodies seems to be quite constant, three pairs at one end and two at the other (fig. 93). In the very late prophase, after the chromosome is on the spindle, it is still loosely. organized, and its several characteristic components may be seen (fig. 94). In full metaphase the chromosome becomes more compact, and, as I have described, usually consists of a central mass with a small component at each side. Occasionally one finds the chromosome in metaphase with two small elements on one side and one on the other (fig. 95). In early anaphase, the smaller components appear as knobs or strands projecting at an angle from the main mass (figs. 89, 92 B and 96).

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[2] Professor Wilson has called my attention to his description of the X chromosome in Lygaeus bicrucis ('12), where in the growth period it consists of several, usually three, paired segments, somewhat resembling the X chromosome of N. indica.

In the second division, the peculiar X Y pair take up the usual position in the center of the spindle surrounded by a ring of 12 other chromosomes (figs. 97, 98 A, B, C and 99 A, B). In the group are the double chromosomes, lying on the spindle with the split, which was in evidence in the first division, on the equatorial plane, marking the line of separation of its components. There is also present in this division an asymmetric quadripartite chromosome, similar to the d-chromosome described by Wilson ('11) in Nezara (figs. 97, 98 C and 99 B). The appearance of the X chromosome in the second division is somewhat variable, owing probably to the way it has been cut in the section; it most often appears as a central mass with small masses hanging down from each end, Y being usually attached by a fiber to one of these ends (figs. 98 B, 99 A and 100 B). The fact that its shape is not dependent on the presence of Y is shown by its reversed position in some cases (fig. 100 E). The connection between the different parts of X is in many cases not evident, but this may be due to the thinness of the thread or to its being just without the plane of section (fig. 97 and 100 C, D). The characteristic ring figure with X Y in the center is seen in polar views (figs. 101 and 102). Among the chromosomes in the peripheral ring are the two large ones which appeared double in side views; their double nature usually does not show now or is only indicated by a notch in the side. Owing to the peculiar shape of X, detached pieces of it often appear in polar view, and since these are about the same size as Y, it is impossible to tell which these pieces are; in figure 102, the three parts in the center are parts of the X Y pair. Figure 103 shows the different appearances of X Y in polar view. In anaphase, X is usually slow in going to the pole, and is found stretched between the two groups of chromosomes (fig. 104). More frequently X appears very much as it did in the first anaphase, as a central mass with smaller pieces attached, often by the merest thread, or with no visible connection (figs. 105 and 106); it will be observed that these end pieces may extend either toward or away from Y. Sister groups from an anaphase in polar view show again the number and size relations of the chromosomes, and are practically identical, except for the central component, X or Y (fig. 107). The chromatin content of the two classes of spermatozoa differs more in this species than in any of the others, owing to the unusually large volume of X. The structure of X in telophase is very similar to its previous structure, and it is frequently difficult to discover any connection between its components (figs. 108 and 109).

In the spermatogonial (figs. 110 to 113) and the oogonial (figs. 114 and 115) groups, there are 26 chromosomes. Especially characteristic are the long winding chromosomes, single in the male and paired in the female groups; this is of course the X chromosome. Although X shows such a marked tendency to break up into smaller parts in the spermatocyte divisions, I have found little indication of this in the diploid groups. I have examined many groups, and have always found X represented by a single chromosome of almost equal width throughout its length, with sometimes a slight tendency toward notching at one or both ends. In both male and female groups the two large double chromosomes of the spermatocyte divisions are represented by the four next largest chromosomes, and the two small ones by four little ones.

b. The X chromosome of N. indica

The characteristic and unusual appearance of the X chromosome as described in the preceding section leads one to suspect that it is really compound, and is in the process of splitting off or uniting with smaller chromosomes. Whether these smaller pieces ever become entirely separated from the chromosome proper in N. indica, it is difficult to tell. They are often attached only by a thin fiber, no thicker than the fiber connecting the chromosome with its mate Y, and they often appear to be entirely separate. If these pieces are separate in N. indica, or if they eventually become separate in this species or in one derived from it, X may be considered as a complex of chromosomes rather than as a single chromosome. This complex is however, represented in the diploid groups by a single chromosome. N. indica is, therefore the first link in the chain, from a simple X which occurs in many forms either with or without a mate Y, through those cases where X is composed of two elements sometimes combined, sometimes separate (Phylloxera, Agalena, Syromastes), to the cases where X consists of two or more elements always separate, but acting as a unit in the maturation divisions, e.g., Thyanta calceata (Wilson '11), Galgulus and some of the reduvioids (Payne '09), Ascaris lumbricoides (Edwards '10) and finally Ascaris incurva, where X consists of eight elements (Goodrich '14). Perhaps the case next in series after N. indica is Phylloxera caryaecaulis, where the two parts of X are very unequal, and are indistinguishably fused during part of their history. We have additional evidence from N. indica for Wilson's ('11) assumption that X is essentially compound in nature with a tendency for the parts to segregate as separate chromosomes, and strong morphological evidence for sex-linked characters.

It is also possible that the small pieces of the X chromosome represent the starting point in the appearance of supernumerary chromosomes, such as are found in Banasa calva (Wilson '07), Metapodius (Wilson '07, '09), the Diabroticas (Stevens '08), and Ceuthophilus (Stevens '12). These chromosomes divide in only one of the maturation divisions, as one should expect if they have originated from X, and they frequently accompany the XY pair in some forms. Other hypotheses have been suggested for the origin of the supernumeraries; by the passage of both X and Y to the same pole (Wilson '09), by the replacement of Y by a second m-chromosome (Wilson '10) and by an abnormal division of the X chromosome in one of the maturation divisions (Stevens '12).

Reference should be made to the XY chromosome as described by Stevens ('07) for Drosophila, where there is a third element which looks as though it were separating from the X Y pair in the maturation divisions, although no trace of this was to be found in the diploid groups. Metz ('14), however, who has studied the chromosomes of Drosophila carefully, says that he finds no indication of such a separate element in the X Y pair.

VII. COMPARISON OF THE CHROMOSOMES IN THE SIX SPECIES

The chromosome groups of the different species of Notonecta are in general very similar in number, arrangement and size relations. An effort has been made to account for the slight differences which do occur in the different species.

a. Autosomes

The typical arrangement of the chromosomes in the first maturation division of the six species is a ring of chromosomes of varying sizes, surrounding one or two small chromosomes in the center, and in the second division a ring surrounding the XY pair. The only departures from this grouping are in the case of N. shooterii where one small one is usually a little within the peripheral ring in the first division; and in the case of N. indica, in the first division, where two intermediate ones are somewhat within the ring. The arrangement in N. shooterii may be explained on the assumption that the second small chromosome, which is in the center in N. undulata and N. indica, is in the process of shifting its position to the outside ring, possibly a stage preparatory to fusion with the largest or some other chromosome, or the reverse, a stage in separation. If this be true, we have represented in N. shooterii another step in the change in number of chromosomes in the different species, comparable with that represented in N. insulata, as pointed out in my former paper. The divergence from the typical arrangement in N. indica may be explained on the ground that this species is an offshoot from N. undulata, and its chromosome grouping correspondingly a modification of that of N. undulata, indicated by the displacement of two of the chromosomes from the outer ring.

With regard to number, the six species may be grouped into two classes, one class having a diploid number of 26, first spermatocyte number of 14, second spermatocyte number 13, and the other class with a diploid number of 24, first spermatocyte number 13, second spermatocyte number 12. To the first class belong N. undulata, N. shooterii and N. indica; to the second class belong N. irrorata and N. glauca. N. insulata belongs rather to the latter group, though it is somewhat intermediate, since many of the first division cells show 14 chromosomes. The difference in number in the two classes can probably be attributed, as previously suggested, to the presence or absence of a particular small chromosome which, when present, lies in the center of the group in the first division, as in the case of N. undulata and N. indica, or just within the peripheral ring as in N. shooterii. In N. insulata its presence or absence can be directly attributed to its separation from or its fusion with the largest chromosome, and I have attributed its absence in N. irrorata to its permanent fusion with the largest chromosome. This explanation will likewise apply to N. glauca, and I believe that this is the correct explanation. There is, however, another possibility which may apply to both of these cases or to one of them. This is that the second small chromosome in present in the peripheral ring, and one of the other peripheral chromosomes represents two chromosomes of the 14-group. In this event, N. shooterii represents the step in transition, the small chromosome being intermediate in position between the central and peripheral positions. There is a small chromosome in the peripheral ring in both N. glauca and N. irrorata, but there is nothing in its position or behavior to show any relationship with the one in the center in the 14-groups, as is the case in N. shooterii. Moreover, there is a small chromosome in the periphery in N. insulata even when the two small ones are present in the center. It seems therefore more plausible to assume that in N. irrorata and in N. glauca, the fusion of the second small chromosome and the largest one is permanent, and that N. insulata presents one intermediate step and N. shooterii another. Such a process of fusion and separation of chromosomes is analogous with that described in the case of the sex chromosomes referred to under N. indica, and in the case of the sex chromosomes with other chromosomes in the phasmids (Sinéty '01), a few other Orthoptera (McClung '05), one of the mosquitoes (Stevens '11) and Ascaris megalocephala (Boring '09, Boveri '09, Edwards '10). In the different species of Drosophila, Metz ('14) has attributed the difference in number to a separation of certain V-shaped chromosomes into single bars.

In all the species of Notonecta described, there are two chromosomes larger than any of the others, particularly striking as four large ones in the diploid groups. Together with X, which is usually the fifth largest, we find five large chromosomes in the male and six large ones in the female diploid groups. N. irrorata forms an exception in having seven large chromosomes in the spermatogonial groups. When these chromosomes attain an especially large size, they appear in the spermatocyte divisions as large compound chromosomes. These are made up of four curved bars or U's in the first division, so arranged as to form a cross, though this structure is not always evident owing to an overlapping of parts. Two of these bars go to each sister cell, and in the second division, their line of separation lies along the equatorial plane so that one goes to each spermatid. These chromosomes are really typical tetrads whose four parts remain separated until finally distributed in the second division. I have called them double or compound chromosomes because their component parts are distinguishable, giving them a composite appearance, and also because the final element often looks as though it were composed of two equal parts, owing to a notch in the center. Whether this notch is merely a remnant of the original bend in the curved bar, or whether it indicates that the chromosome is in the process of splitting into two equal parts may possibly be determined by work on other species. Since there must be a limit to the size of a chromosome, and since these large ones, especially the largest one in N. glauca, have become quite massive, it seems plausible to suppose that this chromosome is separating into two parts. If so, then the large chromosome of N. insulata is of particular interest since it is composed of a larger and a smaller part and the notch indicates another line of separation. If the unequal parts are in the process of fusion, an equal division of the chromosome means a redistribution of particles in the resulting chromosomes; if the unequal parts are in the process of splitting, then the chromosome is splitting at two points, one split being realized in the first division, the other being only indicated. Various stages in the formation of these large compound chromosomes are to be found in the different species. In the case of the largest chromosome of N. glauca and of the two largest ones in N. indica, the compound nature is evident in the first division. In the case of the largest chromosome of N. insulata and the second large chromosome of N. glauca, the compound nature is not noticeable in the metaphase of the first division except in polar view, but is evident in the second division. In the second largest chromosome of N. insulata and the largest chromosome of N. irrorata, a split is noticeable in some cases in polar view of the first division, but the chromosome does not appear double in the second. In the largest chromosomes of N. shooterii and N. undulata there is no evidence of a compound structure. In general, it may be said that the larger the chromosome is, the greater the tendency is for its component parts to separate.

b. Sex chromosomes

In comparing the chromosome groups of the six species of Notonecta, one of the most striking features is in regard to the XY pair. This pair is almost equal, occasionally quite so in N. shooterii, more unequal in N. glauca, still more so in N. insulata and N. irrorata, very unequal in N. undulata and extremely so in N. indica. This difference in the size relations of X and Y in the different species is caused apparently both by X and Y. In comparison with the other chromosomes, X is the fourth largest chromosome in N. irrorata, the third in N. glauca, N. insulata (probably—the lack of diploid groups prevents an absolute determination), N. shooterii and N. undulata, and is the largest in N. indica. Y is the third smallest in N. undulata, about the fifth in N. irrorata, N. indica, N. insulata and N. glauca, and about the eighth in N. shooterii. A similar progressive difference in the relative size of these chromosomes has been shown to occur in the different species of Euschistus (Wilson '06).

VIII. CORRELATION OF SOMATIC CHARACTERS AND CHROMOSOMES

The diagnostic specific characters of Notonecta used by entomologists are the body size, distance between the eyes, width of the pronotum and size of the scutellum. The coloration and markings are to some extent characteristic of the different species, but there is a great variation in color in certain species from a pure white to an almost black, so that this character is regarded as of little value in the determination of species. In the accompanying table, I have listed the size relations of the different parts in the six species, most of the figures being taken from Bueno ('05). An outline of the number and relations of the chromosomes is also given in the table.

In general a definite number and arrangement of the chromosomes is characteristic of each species, and is therefore probably correlated with the characters of that species. A reference to the table will show, however, that the species cannot be lined up with regard to their chromatin content and with regard to each of the somatic characters with any great agreement. It would seem that the distance between the eyes, which is perhaps the most reliable characteristic for the entomologist, is not correlated with the most striking chromosome difference, that of number. There are two characters, however, in which the 14-chromosome species differ from the 13-group. The species with 14 chromosomes are the small forms—N. undulata, N. indica and N. shooterii—whereas the species with 13 chromosomes are the large forms, N. insulata, N. glauca and N. irrorata. Secondly, there is a physiological difference between the 14-species and the 13: the former have a rapid evolution of the germ cells.

TABLE 1

SPECIES	EYES VERTEX: SYNTHLIPSIS	PRONOTUM WIDTH: LENGTH	SCUTELLUM WIDTH: LENGTH	LENGTH OF INSECT	COLOR	NO. OF CHROMOSOMES		SIZE RELATION AND ARRANGEMENT OF CHROMOSOMES IN 1ST DIVISION	SIZE OF X AND Y COMPARED WITH OTHER CHROMOSOMES	DIPLOID NO. OF CHROMOSOMES	SIZE RELATION OF CHROMOSOMES IN DIPLOID GROUPS
						1st division	2d division				
N. undulata...	2⅓	2	1⅕	10-13 mm.	White or black-and-white	14	13	$a + a + M + M' + X + Y + 8A$ Small one in periphery = Y	X = 3d largest Y = 3d smallest X and Y quite unequal	26	5 large 5 small 16 medium } ♂
N. indica.....	1½-2	1¾	1½	9.4-11 mm.	Black-and-white	14	13	$a + a + \mathrm{m} + \mathrm{m}' + X + Y + 8A$	X = largest (compound) Y = about 5th smallest X and Y very unequal	26	5 large 4 small 17 medium } ♂ 6 large 4 small 16 medium } ♀
N. shooterii...	1⅓	1⅔	1⅓	8-13 mm.	White or black-and-white or brown-and-white	14	13	$a + a + M + M' + X + Y + 8A$	X = 3d largest Y = 6-11th smallest X and Y almost equal	26	5 large 4 small 17 medium } ♂ 6 large 4 small 16 medium } ♀
N. insulata....	1+	2	1⅕	12.6-15.5 mm.	Brown-and-black or almost all brown	14, 13	12	$a + a + \mathrm{m} + M' + X + Y + 8A$ or $a + \mathrm{ma} + M' + X + Y + 8A$ Small one in periphery	X = 3d-5th largest Y = 5th or 6th smallest X and Y fairly unequal		

N. irrorata..	3	2	1¼	12.1-14.4 mm.	Black with brown mottling	13	12	*a* + Ma assumed) + 2M′ + X + Y + 7A 2 small ones in periphery	X = 4th largest Y = 4th or 5th smallest X and Y fairly unequal	24	7 large 6 small 11 medium	♂
N. glauca. .	2-2¼	2	1⅓	13.4-17.2 mm.	Pale yellow or yellow-and black or dark brown	13	12	*a* + Ma(assumed) + M′ + X + Y + 8A Small one in periphery	X = 3d largest Y = 4th-6th smallest X and Y almost equal	24	6 large 4 small 14 medium	♀

Explanation. Vertex = distance between eyes at front. Synthlipsis = distance between eyes at base of head. a = small autosome. *a* = small autosome in center. M = largest chromosome or macrochromosome. Ma = macrochromosome associated with small autosome. M = macrochromosome double. M′= next largest chromosome. A = intermediate autosome.

so that all stages in the spermatogenesis are to be found throughout the summer and fall; in the 13-species, on the other hand, the evolution of stages is very slow, so that at one time the testis is filled with cells in the growth stages, then with dividing cells, and in the late summer only spermatozoa are to be found. Probably as a result of this, the cells of the former species are smaller than the latter. Whether these two characters are really correlated with the chromosome number, or whether it is a mere coincidence can probably be determined by work on other species.

Just what the status of each species is in relation to the others cannot be told at present. I think there is little doubt that N. indica is a species derived from N. undulata, as both the somatic characters and the chromosomes indicate this. N. shooterii is particularly puzzling because it seems primitive with regard to the XY pair and yet it seems to represent a transition stage with regard to the autosomes.

IX. CONSTANCY OF THE CHROMOSOMES

Notonecta adds to the ever increasing mass of evidence that the chromosomes are definite bodies with individual peculiarities and characteristics. Each species is characterized by some special behavior or structure or position of certain of the chromosomes, and this characteristic is constant for all the individuals and all the cells of that species with few exceptions. I would especially emphasize the constancy in the arrangement of the chromosomes. There is always some particular grouping of the chromosomes, different in the two divisions, but characteristic of some particular species.

X. SUMMARY

1) An XY pair of chromosomes is present in all six species, the components dividing separately in the first division and going to opposite poles in the second. The components frequently lie side by side in the second metaphase.

2) The X and Y chromosomes are almost equal in most cells of certain individuals of N. shooterii. They are more nearly

equal in certain individuals of N. glauca than in others. In the other species, these chromosomes are more unequal, and are extremely so in N. indica.

3) The X chromosome of N. indica is a compound chromosome composed of larger and smaller parts.

4) In N. undulata, indica and shooterii, there are 14 chromosomes in the first division and 13 in the second and 26 in the diploid groups. In N. irrorata and glauca, there are 13 chromosomes in the first division and 12 in the second and 24 in the diploid groups. In N. insulata, there are 14 or 13 in the first and 12 in the second.

5) In all the species except N. indica, the chromosomes in the first division are arranged in a ring with the small ones in or near the center. In N. indica, two of the intermediate chromosomes are somewhat within the ring. In all the species in the second division, the chromosomes are arranged in a ring with XY in the center.

6) In N. undulata, indica and in the 14-groups of insulata, there are two small ones in the center of the ring in the first division. In N. irrorata and glauca and in the 13-groups of insulata, there is only one small one in the center. In N. shooterii, there is one small one in the center and another between the center and the periphery. In the 13-groups of N. insulata, the second small one is often found attached to the macrochromosome.

7) Large double chromosomes are characteristic of N. insulata, glauca and indica.

8) The differences in somatic characters in the different species cannot be definitely correlated with the differences in chromosome number or arrangement. However, the 14-chromosome species are the smaller species, while the 13-chromosome species are the larger ones.

LITERATURE CITED

Boring, A. M. 1907 A study of the spermatogenesis of twenty-two species of Membracidae, etc. Jour. Exp. Zool., vol. 4.

1909 A small chromosome in Ascaris megalocephala. Arch. f. Zellforsch., Bd. 4, S. 120.

Boveri, Th. 1909 Uber 'Geschlechtchromosomen' bei Nematoden. Arch. f. Zellforsch., Bd. 4, S. 132.

Browne, E. N. 1913 A study of the male germ cells in Notonecta. Jour. Exp. Zool., vol. 14, p. 61.

Bueno, J. R. de la T. 1905 The genus Notonecta in America north of Mexico. Jour. N. Y. Entomol. Soc., vol. 13, p. 143.

Edwards, C. L. 1910 The idiochromosomes in Ascaris meg. and Ascaris lumb. Arch. f. Zellforsch., Bd. 5.

Goodrich, H. B. 1914 The maturation divisions in Ascaris incurva. Biol. Bull., vol. 27, p. 147.

Kirkaldy, G. W. 1897 Revision of the Notonectidae. Trans. Entomol. Soc. London.

McClung, C. E. 1905 The chromosome complex of orthopteran spermatocytes. Biol. Bull., vol. 9, p. 304.

1908 Cytology and taxonomy. Kansas Univ. Bull.

1914 A comparative study of the chromosomes in orthopteran spermatogenesis. Jour. Morph., vol. 25, p. 651.

Metz, C. W. 1914 Chromosome studies in the Diptera. Jour. Exp. Zool., vol. 17, p. 45.

Montgomery, T. H. 1901 A study of the chromosomes of the germ cells of Metazoa. Trans. Amer. Phil. Soc., vol. 20, p. 154.

1906 Chromosomes in the spermatogenesis of Hemiptera Heteroptera. Trans. Amer. Phil. Soc., vol. 21, p. 97.

Morgan, T. H. 1909 A biological and cytological study of sex determination in phylloxerans and aphids. Jour. Exp. Zool., vol. 7, p. 239.

1912 The elimination of the sex chromosomes from the male-producing eggs of phylloxerans. Jour. Exp. Zool., vol. 12, p. 479.

Pantel, J., et Sinéty, R de. 1906 Les cellules de la lignée mâle chez le Notonecta glauca. La Cellule, t. 23, p. 89.

Payne, F. 1909 Some new types of chromosome distribution and their relation to sex. Biol. Bull., vol. 16, p. 119.

Sinéty, R. de 1901 Recherches sur la biologie et l'anatomie des phasmes. La Cellule, t. 19.

STEVENS, N. M. 1907 A study of the germ cells of certain Diptera, with reference to the heterochromosomes and the phenomena of synapsis. Jour. Exp. Zool., vol. 5, p. 359.

1908 The chromosomes in Diabrotica vittata, etc. Jour. Exp. Zool., vol. 5.

1911 Further studies on the heterochromosomes in mosquitoes. Biol. Bull., vol. 20, p. 109.

1912 Supernumerary chromosomes and synapsis in Ceuthophilus. Biol. Bull., vol. 22.

WALLACE, L. B. 1909 The spermatogenesis of Agalena naevia. Biol. Bull., vol. 17, p. 120.

WILSON, E. B. 1906 Studies on chromosomes III. The sexual differences of the chromosome groups in Hemiptera, etc. Jour. Exp. Zool., vol. 3.

1907 Note on the chromosome-groups of Metapodius and Banasa. Biol. Bull., vol. 12. .

1909 Studies V. The chromosomes of Metapodius, a contribution to the hypothesis of the genetic continuity of the chromosomes. Jour. Exp. Zool. vol. 6.

1911 Studies VII. A review of the chromosomes of Nezara, with some more general considerations. Jour. Morph., vol. 22, p. 71.

1912 Studies VIII. Observations on the maturation phenomena in certain Hemiptera, etc. Jour. Exp. Zool., vol. 13, p. 345.

PLATE 1

EXPLANATION OF FIGURES

N. undulata, N. irrorata, N. insulata. × 2000

1 N. undulata, metaphase of the first division, polar view, showing 14 chromosomes, two small ones in the center.

2 N. undulata, metaphase of the first division, side view, showing linear arrangement of the central pairs.

3 N. undulata, metaphase of the second division, polar view, showing 13 chromosomes, XY in the center.

4 N. undulata, metaphase of the second division, side view.

5 A, B N. undulata, sister anaphase groups of the second division, from the same spindle.

6 N. undulata, spermatogonial groups showing 26 chromosomes.

7 N. irrorata, metaphase of the first division, polar view, showing 13 chromosomes, one small one in the center.

8 N. irrorata, metaphase of the first division, side view.

9 N. irrorata, metaphase of the second division, polar view, showing 12 chromosomes. Both X and Y appear in the center.

10 N. irrorata, metaphase of the second division, side view.

11 A, B N. irrorata, sister anaphase groups of the second division from the same spindle.

12 N. irrorata, spermatogonial group, showing 24 chromosomes.

13 N. insulata, metaphase of the first division, showing 14 chromosomes, two small ones in the center.

14 N. insulata, metaphase of the first division, showing 13 chromosomes, one small one in the center.

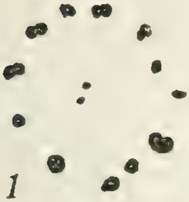
15 N. insulata, metaphase of the first division, side view, with two small ones in the center.

16 N. insulata, metaphase of the first division, side view, with one small one in the center and the large chromosome with a small one attached.

17 N. insulata, metaphase of the second division, showing 12 chromosomes.

18 N. insulata, metaphase of the second division, side view.

19 A, B N. insulata, sister anaphase groups of the second division, from the same spindle.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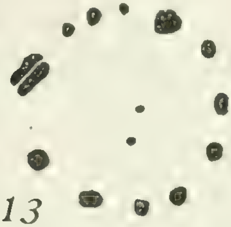
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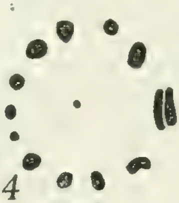
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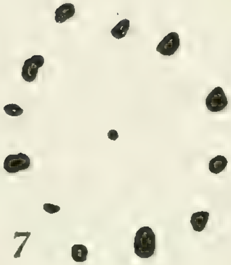
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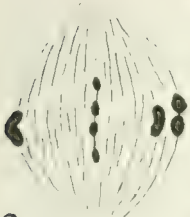
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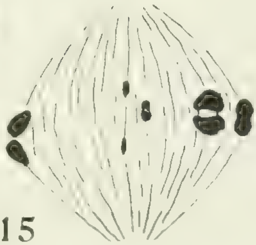
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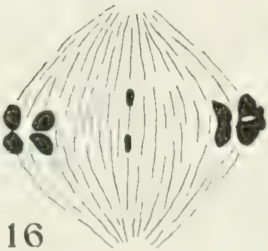
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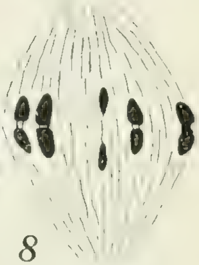
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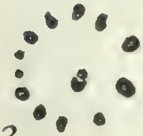
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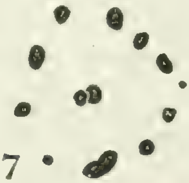
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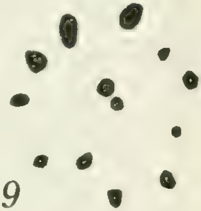
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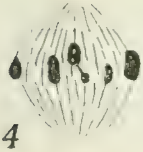
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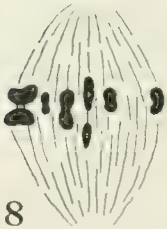
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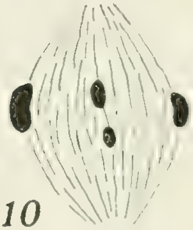
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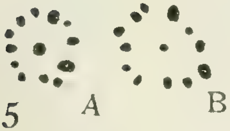
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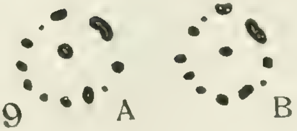
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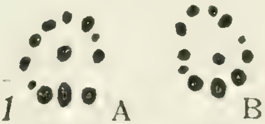
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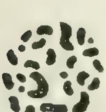
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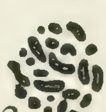
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PLATE 2

EXPLANATION OF FIGURES

N. glauca, first division. × 2000

20 to 22 Polar views of the metaphase, showing 13 chromosomes.

23 Side view of initial anaphase.

24 A, B, C Serial sections of entire spindle in side view, showing 13 chromosomes.

25 B Side view showing components of central dyad on different fibers. A. Other chromosomes from the same spindle.

26 Large compound chromosome in side view at the side of the spindle. The dark and light portions represent similar dark and light areas on the chromosomes.

27 Large compound chromosome in side view on the top of the spindle.

28 Large compound chromosome in polar view.

29 and 30 Early anaphase, side view, showing division of the large compound chromosome.

31 and 32 Later anaphase, side view, showing both large chromosomes.

33 Polar view of anaphase.

34 Late anaphase, showing chromatic substance from the large chromosome streaming down over the spindle fibers.

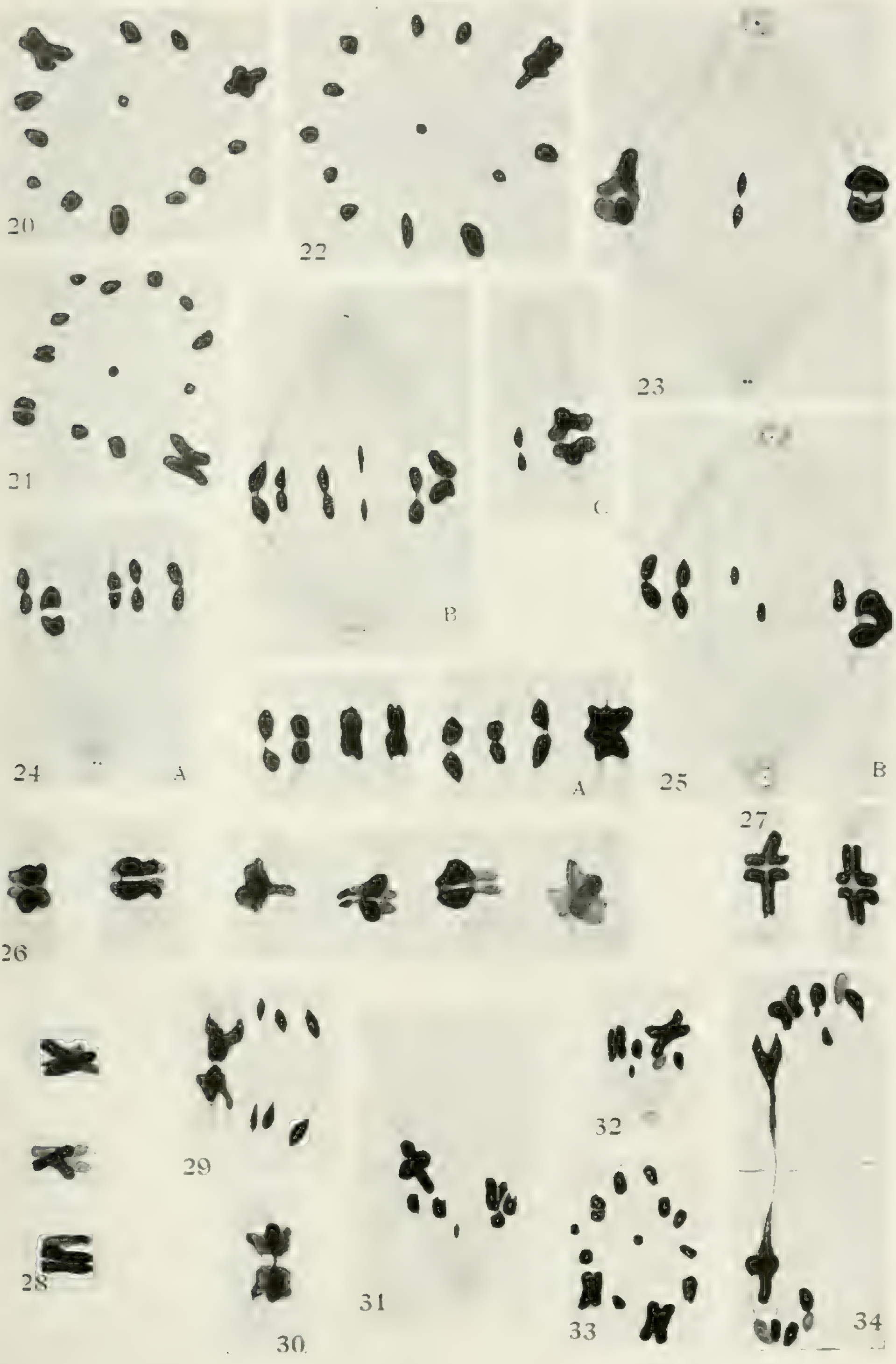
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PLATE 3

EXPLANATION OF FIGURES

N. glauca, second division. × 2000

35 Telophase of the first division, prophase of the second, showing both large chromosomes.

36 Metaphase, side view, showing large compound chromosome and XY.

37 A, B, C Serial sections of entire spindle in side view, showing complete set of 12 chromosomes.

38 A, B, C Same, only X and Y are on different fibers.

39 and 40 Polar view, showing 12 chromosomes. Both X and Y appear in the center.

41 Side view of a metaphase spindle, showing two large and two small chromosomes.

42 XY chromosomes from one individual.

43 XY chromosomes from another individual, showing greater inequality of the two chromosomes.

44 Early anaphase showing large chromosomes and XY.

45 Late anaphase, showing two large chromosomes near upper pole and largest one near lower pole.

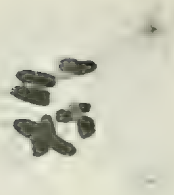
46 Late anaphase, showing largest chromosome near pole.

47 A, B Sister anaphase groups from same spindle, central chromosomes (X and Y) considerably unequal.

48 A, B Same. X and Y more nearly equal.

49 Spermatogonial group, showing large chromosomes and small ones.

50 Oogonial group showing 24 chromosomes.



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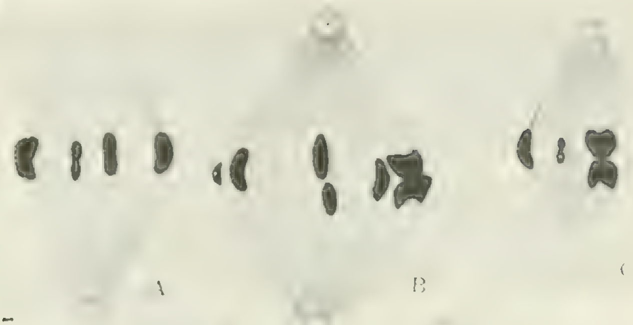

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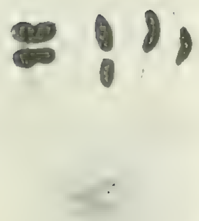

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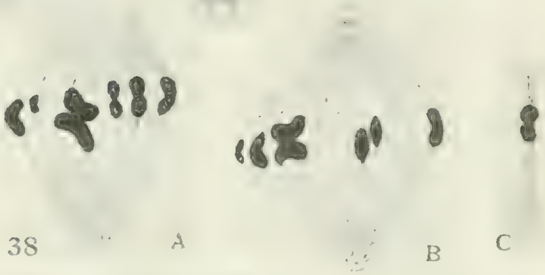

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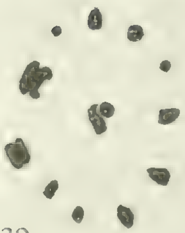

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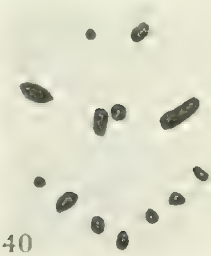

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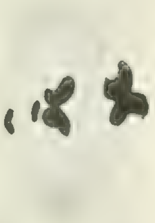

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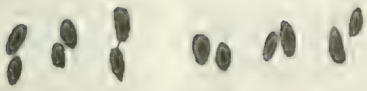

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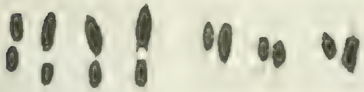

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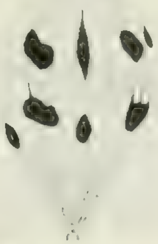

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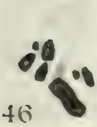

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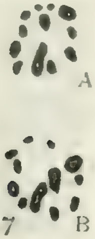

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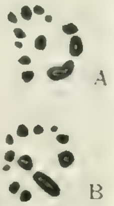

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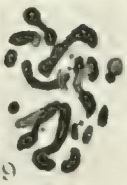

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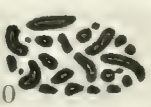

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PLATE 4

EXPLANATION OF FIGURES

N. shooterii, first division. × 2000

51 Polar view, first metaphase, showing 14 chromosomes, one small central one and one just within the peripheral ring.

52 Same, second small one further inside the ring.

53 Side view of metaphase, two small ones at the same focus.

54 A, B, C Serial sections of entire spindle in side view, showing all the chromosomes. In B, the second small chromosome lies at a lower level than the large ones.

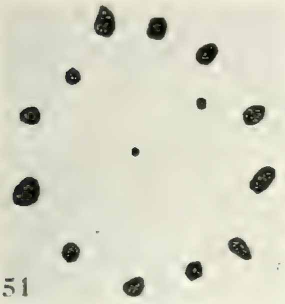
55 Oblique section, showing the second small chromosome within the peripheral ring.

56 Side view of metaphase, showing components of central chromosome on different fibers, and the second small chromosome nearer the center than the large ones.

57 A, B Serial sections of entire spindle in side view of late anaphase, showing all the chromosomes in each daughter group. In B, the two small chromosomes are shown in proximity.

58 to 60 Side views of late anaphase groups, showing proximity of the two small chromosomes.

61 Polar view of late anaphase, showing the two small chromosomes in the center.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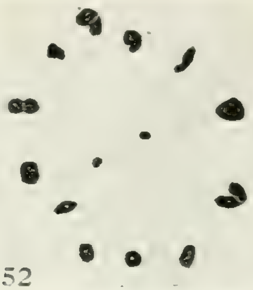
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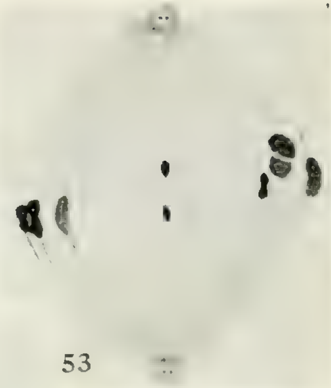
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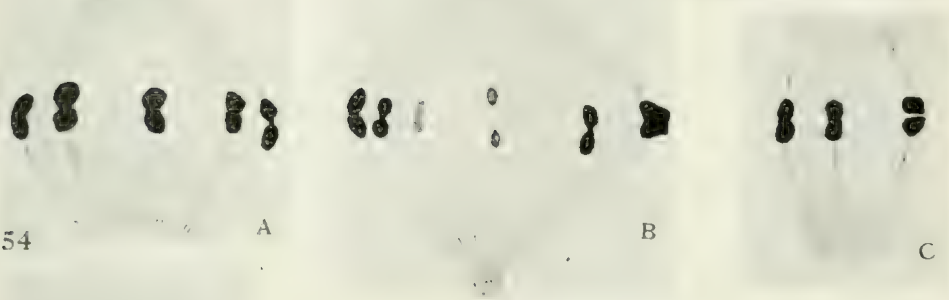
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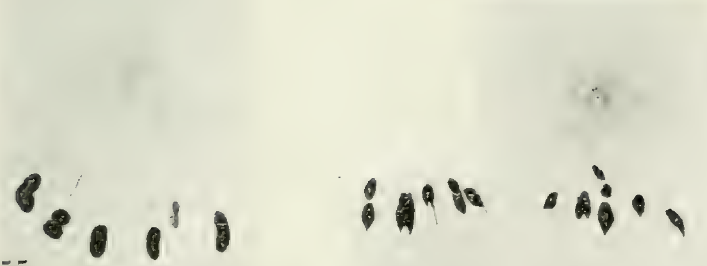
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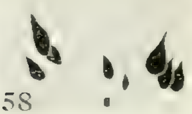
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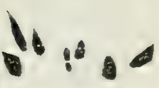
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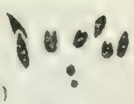
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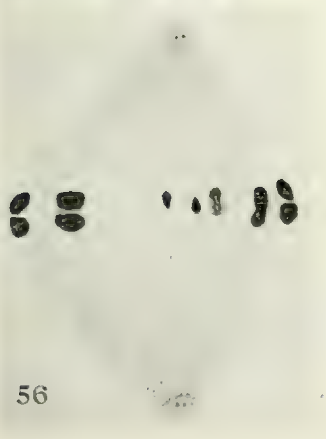
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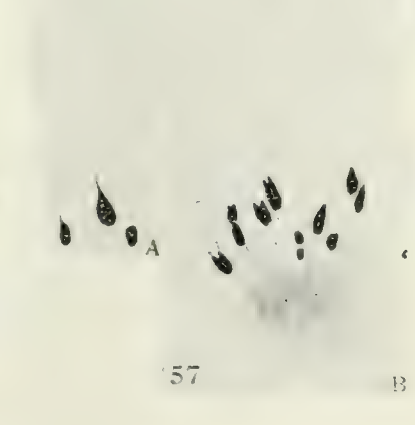
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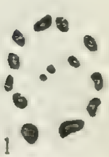
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PLATE 5

EXPLANATION OF FIGURES

N. shooterii, second division. × 2000

62 A, B, C Serial sections of entire spindle in side view, showing all 13 chromosomes.

63 and 64 Polar view of metaphase, showing 13 chromosomes.

65 A, B Serial sections of entire spindle, XY lying side by side.

66 Side view of anaphase.

67 A, B Sister groups of early anaphase.

68 The XY pairs from one individual, showing marked inequality.

69 and 70 The XY pairs from two other individuals, showing less inequality.

71 to 74 The XY pairs from four other individuals, showing still less inequality and in some cases an apparent equality.

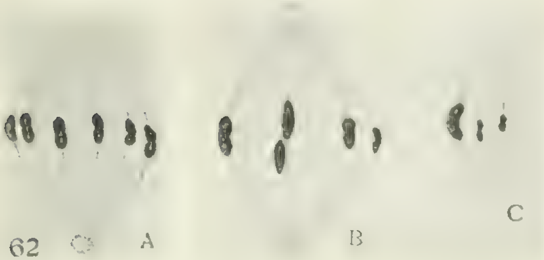
75 A, B Sister anaphase groups from the same spindle, X and Y markedly unequal.

76 A, B Same, X and Y more nearly equal. (A small chromosome is missing in A at the upper left hand corresponding with the one in B.)

77 A, B Same, X and Y practically equal, making the two groups almost identical.

78 and 79 Spermatogonial groups, showing 26 chromosomes including five large ones.

80 Oogonial group, showing 26 chromosomes including six large ones.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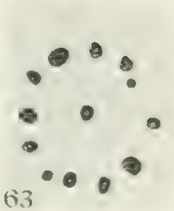
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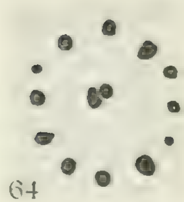
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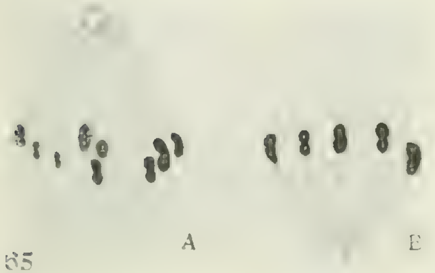
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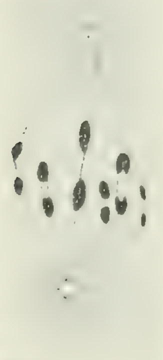
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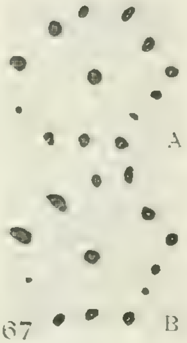
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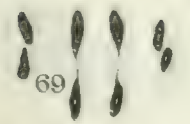
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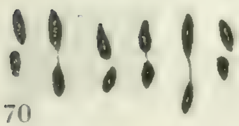
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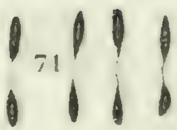
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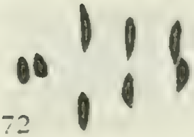
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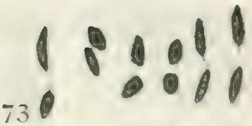
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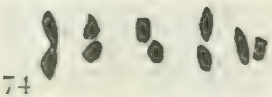
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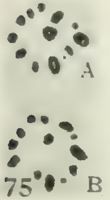
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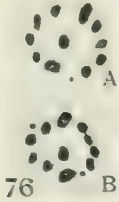
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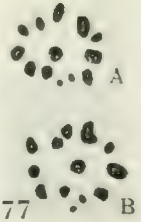
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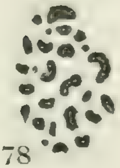
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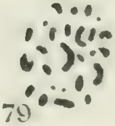
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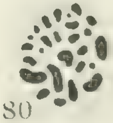
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PLATE 6

EXPLANATION OF FIGURES

N. indica, first division. × 2000

81 Side view of metaphase, showing two small chromosomes in the center; the chromosome in light grey is at the same focus as the central ones, and therefore within the peripheral ring.

82 Same, only the two central pairs are linearly arranged. The X chromosome is shown at the left and a large double at the right. The light and dark portions represent similar light and dark areas seen on the chromosomes. The chromosome in light grey is within the peripheral ring.

83 and 84 Side views showing X and one double chromosome.

85 A, B, C Serial sections of entire spindle, showing all the 14 chromosomes; two large doubles in A, two small central ones and another chromosome in light grey at the same focus in B, and X in C.

86 to 88 Polar views of the metaphase, showing 14 chromosomes.

89 Side view of anaphase showing division of X.

90 and 91 Late anaphase, showing X and large chromosomes in sister cells; both large ones are shown in 91.

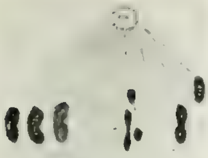
92 A, B, C Serial sections of entire spindle in anaphase, showing all the chromosomes in both sister cells.

93 X chromosome in early prophase, before the spindle is formed.

94 X chromosome in very late prophase, after the spindle is formed.

95 X chromosome in metaphase.

96 X chromosome in anaphase.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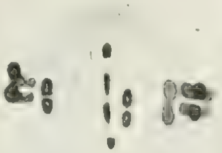
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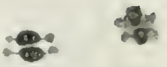
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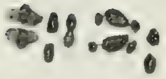
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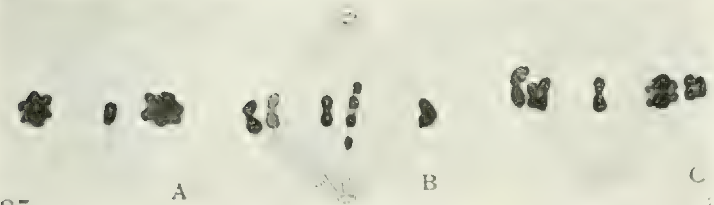

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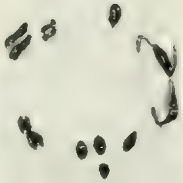
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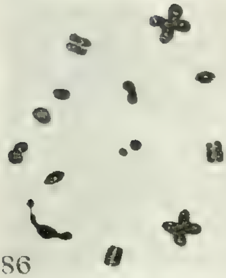
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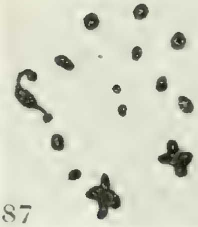
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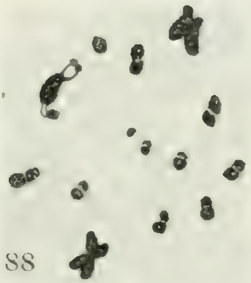
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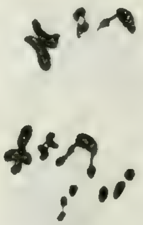
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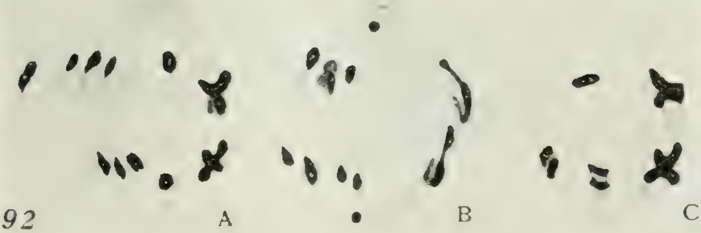

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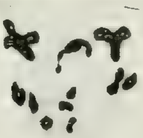
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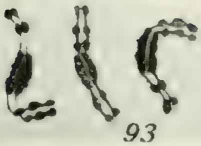
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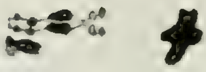
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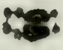
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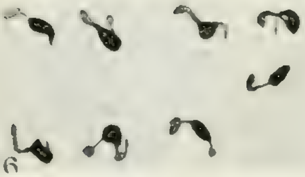
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PLATE 7

EXPLANATION OF FIGURES

N. indica, second division. × 2000

97 Side view of metaphase, showing X and Y, one large double chromosome and an asymmetric tetrad.

98 A, B, C and 99 A, B Serial sections of two entire spindles, showing all 13 chromosomes.

100 A to E The XY chromosomes in side view of the metaphase.

101 and 102 Polar views of metaphase, showing 13 chromosomes. The three bodies in the center in 102 are parts of XY.

103 The XY chromosomes in polar view of the metaphase.

104 Side view of spindle in anaphase, XY in the center.

105 The XY chromosomes in early anaphase.

106 The XY chromosomes in late anaphase.

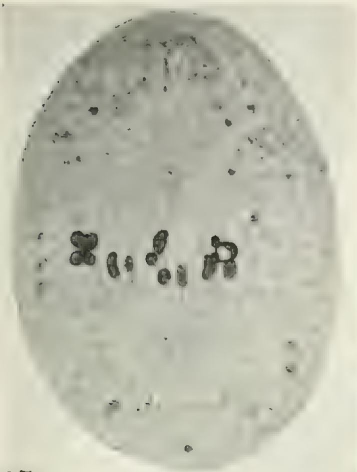
107 A, B Sister anaphase groups from same spindle, A containing X and B containing Y, otherwise practically identical.

108 Side view of early telophase, showing X near upper pole.

109 The X chromosome in side view in telophase.

110 to 113 Spermatogonial groups, showing 26 chromosomes including five large ones; X is the largest.

114 and 115 Oogonial groups, showing 26 chromosomes including six large ones; the two largest are the two X chromosomes.



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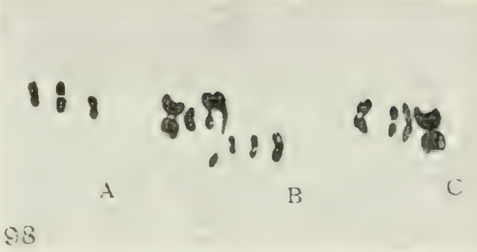

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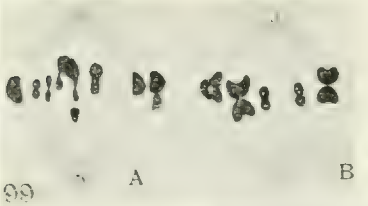

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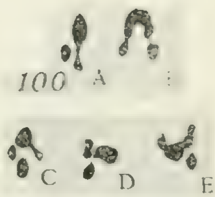

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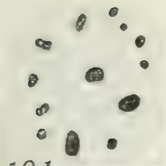

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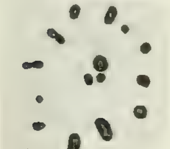

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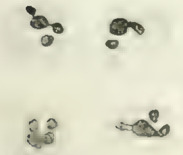

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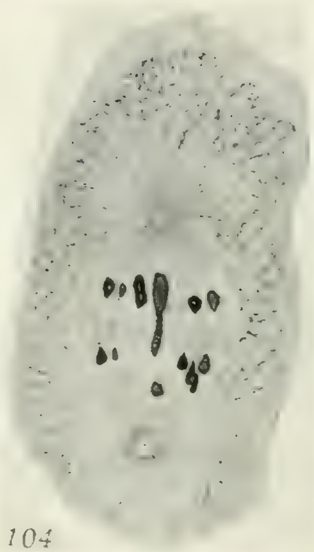

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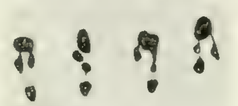

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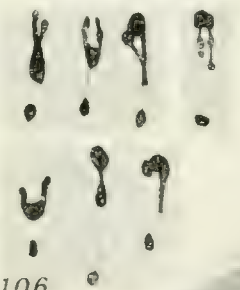

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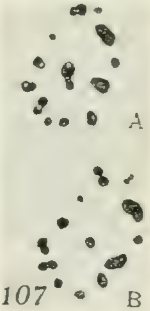

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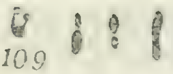

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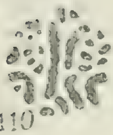

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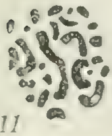

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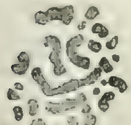

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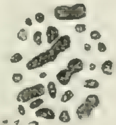

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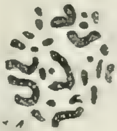

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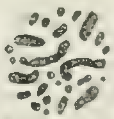

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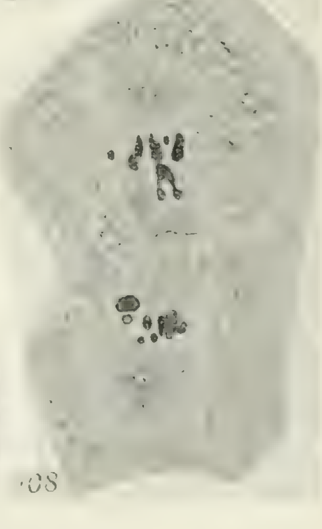

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IX.

A PHYSIOLOGICAL STUDY OF SPECIFIC GRAVITY AND OF LUMINESCENCE IN NOCTILUCA, WITH SPECIAL REFERENCE TO ANESTHESIA.

BY ETHEL BROWNE HARVEY,

Formerly of Cornell University Medical School, New York City.

[Extracted from Publication No. 251 of the Carnegie Institution of Washington, 1917. Pages 235 to 253.]

A PHYSIOLOGICAL STUDY OF SPECIFIC GRAVITY AND OF LUMINESCENCE IN NOCTILUCA, WITH SPECIAL REFERENCE TO ANESTHESIA.

By Ethel Browne Harvey.

INTRODUCTION.

The present study has been chiefly concerned with the specific gravity and the effects of anesthetics on the luminescence of *Noctiluca.* The effects of other substances—acids, alkalies, KCN, etc.—have also been studied, as well as the action of electricity, change of osmotic pressure, temperature, etc., in order to gain an accurate knowledge of the more general physiological aspects of luminescence in this form. Some observations on these subjects were made by Quatrefages in 1850, Krukenberg in 1887, and Massart in 1893, but most of the work is too old and the methods too crude to be of much value, since definite concentrations were not considered nor the results interpreted from the physico-chemical aspect.

The results were obtained at the Marine Biological Laboratory of the Imperial University of Tokyo, situated at Misaki, Japan, where the great abundance of *Noctiluca* offered unusual advantages. The animals occur in such quantities that a layer an inch thick on the surface of a large aquarium jar can soon be collected, and on dark nights the sea is extremely brilliant in any area where it is disturbed, especially where the waves lap the shore. It gives me pleasure to thank Professor I. Ijima for the facilities of the laboratory, and also Professor C. Ishikawa for his many kindnesses during my stay there.

LIGHT RESPONSE IN NOCTILUCA.

As is well known, noctilucas, when undisturbed and under perfectly normal conditions, are not luminous; but when disturbed or stimulated in any way, they give a bright flash of a distinctly bluish tinge, lasting only an instant. This is the normal light-response of *Noctiluca.* If, however, the animals are not in good condition, a faint, continuous glow issues from them; such a glow is usually preliminary to the death of the animals, and is also called forth by many experimental conditions. These two are the only light responses of the animals. There is never, under normal conditions, a rhythmic flashing, such as is characteristic of certain species of firefly, nor is it possible, by any chemical or physical means, to call forth a rhythmic flashing comparable with the rhythmic twitching of a muscle in pure NaCl.

CHANGE OF OSMOTIC PRESSURE.

Noctiluca can stand a considerable decrease in osmotic pressure without having its luminous response affected, as indicated in table 1. We may dilute the sea-water with fresh-water in any amount down to a proportion of half and half, and the animals still give a normal response—*i. e.*, they flash on stimulation. The response is not quite so bright as in the control in a half-and-half concentration nor in a mixture of 4 sea-water to 6 fresh-water, but the animals continue giving a normal response for 7 days. In the latter concentration the animals also give a steady glow for the first few minutes, owing doubtless to the dying condition of certain of them. If the amount of fresh water is still further increased, the animals do not flash on stimulation, but give a steady glow, lasting for 8 minutes in a mixture of 3 sea-water to 7 fresh-water and of 2 sea-water to 8 fresh-water; and for 5 minutes in 1 sea-water to 9 fresh-water, and for about 3 minutes in pure fresh-water. When the osmotic pressure is increased by concentrating the sea-water to half its volume, a steady glow is given for more than 20 minutes.

TABLE 1.—*Effect of diluting sea-water with fresh-water.*

Ratio of sea-water to fresh-water.	Luminous response.	Specific gravity.
9 to 1	Normal, 10 days (experiment discontinued).	Float.
8 to 2	Normal, 10 days (experiment discontinued).	Float.
7 to 3	Normal, 9 days	Float.
6 to 4	Normal, 9 days	Distributed through water 10 minutes; then float.
5 to 5	Normal, 7 days (poor)	Most sink in 15 minutes; float after 20 minutes.
4 to 6	Normal, 7 days (poor), constant glow 3 minutes at start.[1]	Sink in 15 minutes; float after 1 hour.
3 to 7	Constant glow 8 minutes	Sink.
2 to 8	Constant glow 8 minutes	Sink.
1 to 9	Constant glow 5 minutes	Sink.
Fresh-water.	Constant glow 3 minutes	Sink.

Especially interesting results have been obtained in this series of experiments with regard to specific gravity. Normally, noctilucas are less dense than sea-water, so that when placed in an aquarium jar they soon rise to the top, where they form a layer somewhat pinkish in color. Unlike the floating siphonophores, there are no air-bubbles, and unlike certain pelagic eggs, there are no large oil-drops in the animals, so that their lower specific gravity must be due to the fact that their salt-content is less than that of sea-water. When placed in sea-water concentrated to half its volume they shrink, and when the sea-water is diluted with fresh-water they swell. The plasma membrane, therefore, shows the usual semipermeability to the balanced salts of sea-water—*i. e.*, impermeability to the salts and permeability to water. Out in the open bay, although most of the animals are at the surface on a calm night, many can be seen through a glass-bottom bucket well below the surface. Moreover, on windy days, they are not found at the surface, indicating that they have the power of increasing their specific gravity and sinking, owing to a temporary increase in permeability which allows the salts of sea-water to pass in or water to pass out. When noctilucas die they immediately sink, the permeability having been increased, causing an increase in specific gravity.

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[1] The constant glow at the start is without doubt due to the dying condition of some of the animals.

That these animals can also decrease their specific gravity is shown by the following facts. In all dilutions of sea-water with fresh-water down to 6 sea-water to 4 fresh-water, they rise immediately to the top, becoming and remaining somewhat swollen for more than 10 days. These dilutions are still of greater density than the animals. In a mixture of 6 sea-water to 4 fresh-water they remain distributed through the water for about 15 minutes, and then rise to the top; this mixture is of approximately the same density as the noctilucas. In a mixture of half sea-water and half fresh-water and also in a mixture of 4 sea-water to 6 fresh-water, the animals sink at first, their salt-content being now greater than that of the surrounding medium, but during the next hour they gradually swell and rise to the top. The process is wholly independent of movement of the tentacle. If water were merely absorbed by the animals until their concentration was the same as that of the surrounding medium, the animals would remain suspended through the liquid; but since they eventually float, they must keep on absorbing pure water until their salt-content and hence their specific gravity is again *less* than that of the new medium (5 sea-water to 5 fresh-water or 4 sea-water to 6 fresh-water), thus reestablishing their normal relation to their surrounding medium. This must involve the absorption of water against the osmotic pressure of the salts of sea-water, a condition contrary to physical laws. This regulatory mechanism is characteristic of living cells only and is not destroyed under any experimental conditions. Anesthetics, acids and alkalies, KCN, and pure salts of sea-water do not affect the regulation, except when they cause irreversible changes and death of the cells. It is quite probable that this peculiar type of osmo-regulation is not characteristic of noctilucas alone, but may be possessed by other marine plankton forms without gas-chambers which float and sink as occasion demands.

The water absorbed by noctilucas in mixtures of 5 sea-water to 5 fresh-water and 4 sea-water to 6 fresh-water accumulates in large vacuoles formed by strands of protoplasm pulling away from the cell-wall and forming membranes around the accumulated liquid. I have observed these vacuoles bursting or being expelled bodily from the cell. In these same concentrations of sea-water an interesting protoplasmic fragmentation also takes place, secondary smaller masses of protoplasm being formed, sometimes isolated and sometimes connected with the main mass by strands of protoplasm. The vacuoles formed by *Noctiluca* in these mixtures of fresh-water with salt-water are perhaps comparable with the contractile vacuoles of *Paramœcium*, *Amœba*, and other fresh-water forms, where its function is likewise osmoregulatory. It is true that in general in salt-water protozoa no contractile vacuoles are present, since the osmotic pressure of sea-water balances that of the animal and no osmoregulatory mechanism is needed. When *Noctiluca* is put under conditions where it must rid itself of a certain amount of fresh-water, similar though less perfect vacuoles can be formed.

In still more dilute mixtures of sea-water and fresh-water, the animals absorb water until they burst and then shrink; they are killed and sink to the bottom. That this is an osmotic effect and not due to the mere dilution of salts is shown by diluting the sea-water with isotonic cane-sugar, thus diminishing the salt-content but not the osmotic pressure. After testing various concentrations of cane-sugar to determine which was isotonic, it was found that the animals responded normally longest in m cane-sugar, so that this was used to dilute the sea-water. In m cane-sugar in distilled water the animals give a normal response for 2 hours; this is accompanied during the last hour by a steady glow; but when sea-water is added to the m sugar in various proportions, the effect on the luminescence is only slight (table 2). When 3 parts sea-water are added to 7 parts m sugar or any greater proportion of sea-water is used, the animals give a normal response for more than 9 days. In mixtures of 2 sea-water to 8 sugar a normal response is given for 4 days, and in 1 sea-water to 9 sugar a normal response is given for 2 days. The noctilucas, therefore, like muscle and other cells, must be bathed by a certain minimal salt-content as well as surrounded by a fluid with a certain osmotic pressure. They behave in this respect like the luminous bacteria investigated by E. N. Harvey (1914).

TABLE 2.—*Effect of m cane-sugar+sea-water.*

m cane-sugar to sea-water.	Luminous response.
m sugar........	Normal response 2 hours; constant glow during last hour.
9 to 1	Normal response 2 days.
8 to 2	Normal response 4 days.
7 to 3–1 to 9....	Normal response 9 days (experiment discontinued).

SALTS OF SEA-WATER.

In any one of the pure salts of sea-water, made up in m/2 solution in distilled water, *Noctiluca* ceases to give any luminous response after 2 hours (table 3). $CaCl_2$ is the most toxic, no normal response being given, but a constant glow for 2 minutes. KCl is least toxic, a normal response being given for 2 hours. In NaCl and in $MgCl_2$ there is a normal response for an hour, and this is accompanied on first immersion in the salt by a constant glow which soon passes off, doubtless owing to some of the animals dying. In a mixture of NaCl with one of the other salts, in the proportion occurring in sea-water, the normal response lasts much longer than in any of the pure salts; a little under a day in NaCl+KCl, and 5 days in $NaCl+CaCl_2$ or $NaCl+MgCl_2$.

TABLE 3.—*Effect of salts of sea-water.*

Solution (in distilled water).	Luminous response.
m/2 NaCl	Normal, 1 hour (poor); momentary constant glow at start.[1]
m/2 KCl	Normal 2 hours.
m/2 $CaCl_2$	Constant glow 2 minutes.
m/2 $MgCl_2$	Normal 1 hour; constant glow 10 minutes at start.[1]
m/2 (100 NaCl+2.2 KCl)	Normal, $<$1 day.
m/2 (100 NaCl+2 $CaCl_2$)	Normal, 5 days.
m/2 (100 NaCl+10 $MgCl_2$)	Normal, 5 days.
m/2 (100 NaCl+2.2 KCl+2 $CaCl_2$)	Normal, 5 days.
m/2 (100 NaCl+2.2 KCl+10 $MgCl_2$)	Normal, 7 days.
m/2 (100 NaCl+2.2 KCl+2 $CaCl_2$+10 $MgCl_2$)	Normal, 10 days.
Same mixture+m/2300 NaOH	Normal, 10 days.
Same mixture+m/2300 HCl	Constant glow 15 minutes.

[1] The constant glow at the start is doubtless due to the dying condition of some of the animals.

In a mixture of $NaCl+KCl+CaCl_2$ a normal response is given for 5 days, and in $NaCl+KCl+MgCl_2$ for 7 days. With the four salts in a neutral or alkaline medium (+m/2300 NaOH), the response lasted for more than 10 days, when the experiment was discontinued. The addition of a small amount of acid (m/2300 HCl) to the four salts prohibits any normal response, causing a constant glow for 15 to 30 minutes. (See also under Acids.)

These results showing the injurious effects of pure salts on the luminous response of *Noctiluca* and the improvement on balancing with other salts are in agreement with the general work on the effect of balanced solutions on other physiological activities. The luminous bacteria, on the other hand, are independent of a balanced medium, although they must, like *Noctiluca,* have a certain small proportion of salt in the medium (E. N. Harvey, 1914).

ACIDS AND ALKALIES.

For ascertaining the effects of acids on the luminescence of *Noctiluca*, hydrochloric (a lipoid insoluble) and benzoic (a lipoid soluble) acids were used, the solutions being made up in a neutral artificial sea-water, m/2(100 $NaCl$+2.2 KCl+2 $CaCl_2$+10 $MgCl_2$), so as to avoid the disturbing presence of the buffer phosphates and carbonates.

Certain concentrations of these acids, n/2000 to n/4000 HCl and n/4000 C_6H_5COOH, cause a bright continuous glow lasting from 20 to 60 minutes (table 4). The glow in these solutions becomes gradually brighter, stays at a maximum for some time, and then becomes gradually fainter. In n/4000 HCl the animals also give a normal response—that is, they flash on stimulation while they are giving a steady glow. Whether it is certain animals in the solution that give the one response at the same time that others are giving the other, or whether all of the animals give both responses, it was not possible to determine. Stronger concentrations of these acids kill the animals almost instantly, while weaker concentrations have practically no effect.

TABLE 4.—*Effect of acids and alkalies.*

Solution in artificial sea-water.	Luminous response.	Color change in neutral red.
n/500 to n/1000 HCl	Momentary constant glow.	
n/2000 HCl	Constant glow 30 min. (bright, becomes brighter, then fainter).	
n/4000 HCl	Constant glow and normal response 1 hr.	
n/8000 HCl	Normal 6 days (poor); momentary constant glow at start.[1]	
n/16000 HCl	>6 days (exp. discontinued).	
n/500 to n/2000 benzoic	Momentary constant glow.	
n/4000 benzoic	Constant glow 20 min. (becomes brighter, then fainter).	
n/8000 benzoic	>6 days (poor) (exp. discontinued); momentary constant glow at start.[1]	
n/16000 benzoic	>6 days (poor); (exp. discontinued).	
n/125 NaOH (in Mg-free sea-water)	Constant glow 2 min	4 min.
n/250 NaOH (in Mg-free sea-water)	Constant glow 2 min	10 min.
n/500 NaOH	Normal response 30 min., then constant glow 30 min.[2]	1 to 1½ hr.
n/1000 to n/2000 NaOH	Normal >7 days (exp. discontinued)	None.
n/250 NH_4OH	Constant glow 30 seconds	25 sec.
n/500 NH_4OH	Constant glow 75 seconds	30 sec.
n/1000 NH_4OH	Constant glow 4 min. (faint)	1 to 2 min.
n/2000 NH_4OH	Normal 3 min., then constant glow 6 min.[2]	None.
n/4000 NH_4OH	Normal 5 days	None.
n/8000 NH_4OH	Normal >7 days (exp. discontinued)	None.

[1] The constant glow at the start is without doubt due to the dying condition of some of the animals.

[2] The normal response at the start is doubtless due to the delay in taking effect.

The alkalies NaOH (lipoid insoluble) and NH_4OH (lipoid soluble) have an effect upon luminescence similar to that of the acids, except that the constant glow is more faint and of shorter duration; n/125 to n/250 NaOH and n/250 to n/1000 NH_4OH (the first two solutions were made up in Mg-free sea-water, all the others in artificial sea-water) cause a constant glow lasting from 30 seconds to 4 minutes, and no normal response is given (table 4). In slightly weaker solutions, n/500 NaOH and n/2000 NH_4OH, a normal response is given for a short period, owing doubtless to a delay in penetration, followed by a constant glow.

In one series of experiments comparison was made of the penetration time of the alkali and its effect on luminescence. The comparison was made on the same lot of animals stained with neutral red, which is not harmful to them. One set was observed in the dark for the effect on luminescence, while the other set was watched simultaneously by an assistant for color-change. In the case of NaOH the color-change from red to yellow takes place only *after* all light-response has stopped, whether this be a steady glow or a normal response; NaOH, therefore, does not penetrate the cell until after luminescence has ceased. On the other hand, with NH_4OH the color-change takes place *before* the effect on luminescence has stopped—that is, the alkali penetrates the cell while it is still giving a luminous response. For example, in n/500 NH_4OH the color-change takes place in 30 seconds, while the constant glow lasts 75 seconds. These results tally with those of E. N. Harvey (1914), who found that in certain plant and animal cells NaOH penetrates the cell only after its death, while NH_4OH penetrates before the death of the cell. The effect of alkalies on light-production is therefore brought in line with its effect on muscle-contraction, protoplasmic rotation, and other physiological processes.

POTASSIUM CYANIDE.

In strong KCN solutions, m/10 to m/250, a normal response is given at first, lasting from less than a minute to 30 minutes, and this is followed by a constant glow lasting for 7 minutes in m/10 and for 35 minutes in m/250 KCN (table 5). The normal response at the start is probably due to the delay in the KCN taking effect. In a concentration of m/500 the normal response lasts for several hours,

TABLE 5.—*Effect of potassium cyanide.*

Solution in sea-water.	Luminous response.[1]
m/10 (precipitate forms)...	Normal response ¾ minute, then constant glow 7 minutes.
m/60....................	Normal response 4½ minutes, then constant glow 15 minutes.
m/125....................	Normal response 10 minutes, then constant glow 35 minutes.
m/250....................	Normal response 30 minutes, then constant glow 35 minutes.
m/500 to m/2000.........	Normal response >1½ hours, <12 hours.
m/4000 to m/16000.......	Normal response >6 days (experiment discontinued).

[1] The normal response at the start is doubtless due to the delay in taking effect.

and in solutions weaker than m/2000 there is no effect on luminescence. It is of interest to note the slight effect of KCN on oxidations connected with luminescence in comparison with its marked inhibiting effect on oxidations by plant oxidases and on the oxygen consumption of egg-cells and other animal tissues (Loeb and Wasteneys, 1913). The oxidations connected with light-production are apparently entirely different from those concerned with cell-respiration.

ABSENCE OF OXYGEN.

The effect of the lack of oxygen on *Noctiluca* was tested by subjecting the animals to an atmosphere of bubbling well-washed hydrogen from a Kipp generator. When thus treated, the luminescence gradually grows fainter for an hour until it is very faint. If air is now admitted to the tube, the animals respond on irritation as normally and just as brilliantly. Animals caught by the surface film of water on the sides of the tube give light when oxygen is admitted, even without mechanical stimulation. The mere admission of oxygen after its absence may therefore serve as a stimulus.

If the animals are kept for 2 hours, however, in the bubbling hydrogen, the luminescence goes out entirely and does not return on admitting air to the tube, the animals having probably been killed by the treatment. In a control experiment, hydrogen was bubbled through a tube containing the animals, in presence of oxygen; the animals were normal after 1 hour, but gave no response after 2 hours. It is therefore the mechanical disturbance and not the lack of oxygen which is fatal to the animals subjected for 2 hours to the hydrogen.

Light-production in *Noctiluca* is therefore dependent upon a supply of oxygen, as is to be expected. Since the cells deprived of oxygen immediately give light on admitting oxygen, even without stimulation, they must be permeable to oxygen at any time, and not merely upon stimulation and death, when light-production ordinarily takes place. That oxygen is necessary for light-production in *Noctiluca* is contrary to the ideas of Quatrefages (1850), and brings this animal in line with all the other light-producing animals in which the question has been carefully investigated.

TEMPERATURE.

With increase of temperature up to 42° or 43° C., noctilucas give a normal response. From this point to 48° or 49°, a steady glow is given and then the light goes out completely and there is no recovery if cooled immediately. With decrease in temperature, the animals flash more than normally until the temperature reaches 5° to 0°, when they give a constant glow. If kept only a few minutes at 0°, they will recover on warming and again give a normal response; but if kept at 0° for 15 minutes they do not recover.

CENTRIFUGING.

On centrifuging noctilucas for 3 to 5 minutes with a hand centrifuge, the animals form a layer at the surface of the liquid, and in each animal the protoplasm is thrown to the bottom of the cell as the heaviest material. The animals do not orient themselves in any way in the centrifuge, but the protoplasm is thrown to whatever part of the cell happens to be toward the centrifugal pole. After about an hour, many of the animals recover, and the protoplasm may be seen under the microscope returning to its normal position near the mouth-groove and flowing along protoplasmic strands to the periphery of the cell. No separation of the materials of the protoplasm into layers was observed with the centrifugal force used. The effect of centrifuging upon the luminescence is practically *nil*, for the animals give a normal response as soon as taken from the centrifuge tubes and placed in a dish of sea-water.

ELECTRICITY.

When a constant current is passed through a mass of noctilucas, the animals flash brightly at the make, continue glowing during the passage of the current, and cease to glow at the break, giving no flash, but sometimes they stay glowing after the break, and in this case the stronger the current the longer the glow lasts. If stimulated mechanically while the current is passing, they respond by a flash, just as when no current is passing.

The light comes from all parts of the noctiluca and is not restricted to anode or cathode regions. No increase in luminosity could be observed on the cathode side nor decrease on the anode side of the animal comparable with the polar effects of the current on muscle.

The tentacle movement is also influenced by the passage of a constant current. At the make, the tentacle coils up rather tightly, like a watch-spring, and at the break it uncoils, the process being repeated for a number of makes and breaks. This tentacle response is similar to the abnormal behavior of the sartorius muscle of a frog, which sometimes contracts on the make, stays contracted while the current is passing, and relaxes on the break. Spaeth (1916) similarly found that the pigment cells of fish-scales contract on the make, stay contracted during the passage, and relax on the break.

When a mass of noctilucas is subjected to the induced currents of an induction coil they respond with a flash at the break, and at the make also, if the current is strong enough.

If subjected to an interrupted induced current for 45 seconds, the animals flash on the first shock and then remain glowing, but the luminosity becomes gradually fainter. If the current is now stopped for a moment and then passed again, there is again a bright glow. The animals therefore fatigue rather readily when stimulated electrically, as they also do with mechanical stimulation.

RESPONSE OF INJURED CELLS.

If a number of noctilucas are punctured with a needle, causing the cells to collapse, and are then subjected to an interrupted current, they respond just as uninjured cells do. Such punctured and collapsed cells likewise give a normal response when stimulated mechanically—*i. e.*, by pressure of a cover-slip.

Noctilucas are injured by a few shocks of the strong induced current; the protoplasm shrinks away from the cell-wall, leaving a clear area at the periphery. If the animal is further subjected to an induced current, there is again a response, the protoplasmic area becoming luminous, while the clear peripheral area does not. After a short time the cell collapses and the animal appears as a shrunken mass of protoplasm within an irregular membrane. These shrunken animals also respond at the break of a weak induced current with a flash, just as normal cells do; and they likewise give a normal response to mechanical stimulation—*i. e.*, if pressed by a needle. It is thus shown that injury to a noctiluca does not interfere with its response to mechanical or electrical stimulation.

If, however, the injury to the cells is too great, and the cells are completely broken to pieces, they do not respond to stimulation. By pressing a mass of noctilucas through cheese-cloth, a filtrate was obtained containing many empty membranes and fragments of cells, visible under the microscope. This filtrate, although luminous, did not respond to electrical stimulation. Another filtrate obtained by pressing a mass of noctilucas through fine-meshed bolting-cloth, and tested while still luminous, did not respond to electrical stimulation. It is possible, however, that the fact that the noctiluca juice is acid may have some effect on the response in these cases.

LOCATION OF LUMINOUS MATERIAL.

When a noctiluca is giving a bright, constant glow—for instance, when treated with n/2000 to n/4000 HCl—it is fairly easy to observe the light under a microscope in a dark room. The chief luminescence comes from the main mass of protoplasm near the mouth-groove and around the periphery of the cell, while the area between these two regions is much fainter. The luminescence is a general glow over the cell, and a similar glow is suffused momentarily over the cell when a noctiluca is stimulated by a needle. This effect is, however, probably due to a close aggregation of small luminous particles, for when noctilucas are crushed under the microscope and the particles separated by means of a cover-slip, numerous points of light may be observed, similar to stars in the sky. The luminescence, therefore, apparently comes from small granules in the protoplasm, which may be freed from the cell by crushing. That the luminous granules are located in the protoplasm and not elsewhere was shown by stimulating cells injured by electricity. As mentioned previously, a few shocks of an induced current causes the protoplasm of a noctiluca to shrink away from the membrane into a compact irregular mass; when again stimulated, the irregular mass of protoplasm gives light, while the clear peripheral area remains entirely dark. Also, in centrifuged noctilucas there is a gradual motion of luminescence in a cell corresponding roughly with the motion of the protoplasm as it resumes its normal position. These granules in the protoplasm to which the luminescence may be traced do not stain with neutral red nor with methylene blue; there are other granules, however, especially around the periphery of the cell, which do stain with neutral red.

It has not been possible to obtain two substances concerned with light-production in *Noctiluca*, as in *Pholas* (Dubois, 1913, 1914), firefly and *Cypridina* (E. N. Harvey, 1917), etc., one destroyed by heat (photogenin) and the other thermostable (photophelein), notwithstanding repeated efforts to demonstrate them. Only one substance responsible for light-production in *Noctiluca* can be demonstrated, and this substance occurs as granules and burns until it is all used up as soon as it is brought into contact with atmospheric oxygen by crushing the cells; this we may conveniently call photogenin.

ANESTHETICS.

Anesthesia may be defined as a temporary inhibition of some physiological activity and includes a wide range of processes such as cell-division, responses to stimuli of various kinds, light-production, etc. Its chief characteristic is that the condition is reversible—that is, that normal processes are resumed on removal of the anesthetic. The anesthesia may be caused by various means—the constant electric current, change of temperature, and many chemical substances. Among the latter are the salts of Mg, Ca, and other metals, as well as the more common lipoid-soluble anesthetics—chloroform, ether, alcohol, etc. This study deals with the anesthesia of light-production in *Noctiluca* by means of the lipoid-soluble anesthetics.

The only previous work showing the definite anesthesia of light-production in animals is that of E. N. Harvey (1915) on luminous bacteria; he found that the light could be inhibited completely by various alcohols and returned on dilution with sea-water.

Of the various theories of anesthesia, that of Overton (1901) and Meyer (1899) has received widest acceptance. According to this theory, anesthesia is due to lipoid solubility, the anesthetic dissolving in the lipoids of the cell. It has been shown by Overton that there is a direct ratio between the narcotizing power of anesthetics and their lipoid solubility—that is, the more soluble the narcotic in fat, the more narcotic power it has and the smaller the amount necessary for anesthetizing. Just how the solution of the anesthetic in lipoids should change the irritability is not explained by the theory, nor does it take into account other anesthetics which are not lipoid soluble—*e. g.*, neutral salts. No distinction is made between the action of anesthetics in the membrane and in the cell interior. Traube (1913 and 1915) considers anesthesia as due to adsorption of the narcotic by surfaces in the cell, especially colloidal particles, due to the lowering of the surface tension of water. This causes a decrease in catalytic activity of the surface layer, leading to a decrease in the chemical activity of the cell, especially of oxidations. It has been shown that many substances with the same surface tension have the same physiological action, although this is not always true. The fact that dried micro-organisms free of lipoids can be anesthetized supports this theory rather than the lipoid theory (Warburg and Wiesel, 1912). On the other hand, the effect of temperature on anesthesia corresponds more closely with the effect on lipoid solubility than on adsorption of the anesthetic (Meyer, 1901).

That the narcotic acts by interfering with oxidation is held by others besides Traube. Verworn (1913) considers narcosis as an asphyxiation, since narcotized cells behave similarly to those deprived of oxygen. Mathews (1914) explains the relation of oxidation and anesthesia by assuming that protoplasm contains an unstable compound with oxygen, which breaks down on stimulation, and the anesthetic forms a stable compound with oxygen and thus prevents its liberation on stimulation. According to Mansfeld (1909), the anesthetic prevents the oxygen from entering the cell by decreasing the permeability of the surface for oxygen. In favor of these theories is the fact that oxygen consumption by certain cells and in certain oxidations has been shown to be decreased by anesthetics (Warburg, 1911). However, in these cases the concentration necessary to decrease oxidations is much greater than that necessary to produce anesthesia. Moreover, the experiments of Loeb and Wasteneys (1913) on sea-urchin eggs have shown that an anesthetic in the concentration sufficient to prevent cleavage had very little effect on oxidations. A slight decrease in temperature has a much greater effect on the rate of oxidations than an anesthetic of effective strength.

The membrane theory of Lillie (1909–1916) and Höber (1907) is also important. According to this the anesthetic affects the interior of the cell indirectly, the primary effect being upon the permeability of the cell membrane. The change in permeability is supposed to be connected with a change in the aggregation of colloidal particles, protein and lipoid. Lillie's experiments on *Arenicola* larvæ show that the narcotic prevents an increase in permeability, or in some cases causes an actual decrease. Osterhout (1913) has shown also, in *Laminaria*, that anesthetics cause a decrease in permeability, as indicated by an increase in resistance to the electric current. This theory takes into consideration the non-lipoid-soluble as well as the lipoid-soluble anesthetics.

All of these theories fall into two groups: those which consider the action of the anesthetic to be upon the cell-membrane and those which consider the action to be directly upon the cell-contents. Experiments on the anesthesia of light-production in *Noctiluca* have been carried out in the attempt to find out which of these two alternatives is correct.

Although the anesthesia of some processes has been shown to be independent of the consumption of oxygen—for instance, the cleavage of sea-urchin eggs (Loeb and Wasteneys, 1913, and Warburg, 1910)—it would seem probable that the anesthesia of light-production is dependent on oxygen. That oxygen is necessary for light-production in *Noctiluca* has been shown by keeping the cells in an oxygen-free atmosphere, in which case the luminescence stops, but reappears on admitting oxygen. The membrane is freely permeable to oxygen at any time, since those cells deprived of oxygen immediately glow on admission of oxygen. It may be noted in passing that this oxidation is different from that of other cells, as shown by the fact that KCN in relatively high concentrations has no effect, whereas it so quickly affects other oxidations.

The anesthesia must in some way affect the oxidation which is concerned in light-production. Does it do so by preventing oxygen from entering the cell, or does it prevent the utilization of the oxygen already present in the cell? In other words, does the anesthetic act upon the membrane or directly upon the cell interior?

In my experiments various lipoid-soluble anesthetics were tried and the effect of the anesthetic has been in all cases not to prevent light-production altogether, but to prevent a normal response—*i. e.*, a flashing on stimulation. In all the effective concentrations, the animals under the anesthetic produce a steady glow, so faint in some cases that it is not noticeable unless the animals are present in large number. When returned to sea-water, if not left too long in the solution, the steady glow ceases and the normal response returns; this is therefore a reversible phenomenon and a true case of anesthesia. The best concentrations for anesthetizing, as seen by referring to table 6, were: 1/3 saturated chloroform, where the steady glow lasted 2 hours; m/8 ether and m/8 butyl alcohol, steady glow lasting 1½ hours; 1/16 saturated thymol, lasting 1 hour; methyl alcohol, lasting 30 minutes, and 1/4 to 1/8 chloretone, lasting 15 minutes. If returned to sea-water after the period of steady glow, the animals gave no response, the prolonged anesthesia causing death. The tentacle motion was also stopped by the anesthetic in the same concentrations as prevented the normal light-response. The effect on the tentacle was, however, much slower than the effect on light-production, but took place during the early part of the period of constant glow.

The question as to whether the effect of the anesthetic is upon the interior of the cell or upon the cell membrane was tested by destroying membranes, thus allowing oxygen to enter. A number of noctilucas were subjected to m/8 butyl alcohol for an hour in a tube containing sand. Some were then returned to sea-water and recovered, showing that the animals were truly narcotized. The remaining animals were shaken up and broken to pieces by the sand. At the same time, a control lot kept for the same period in sea-water in a tube containing sand were shaken and broken up. A comparison of the two tubes showed that the anesthetized animals were not nearly so bright as the control lot. A microscopic examination of the material showed that the cells were completely broken to pieces into irregular fragments by the sand. A

TABLE 6.—*Effect of anesthetics.*

Solution in sea-water.	Luminous response.	Duration of tentacle movement.
Chloroform:		
Saturated	Constant glow 1 to 2 minutes.	
1/2 saturated	Constant glow 4 to 8 minutes.	
1/3 saturated	Constant glow 2 hours, then normal response, owing to evaporation of chloroform.	30 minutes.
1/4 to 1/8 saturated	Normal response >12 hours (exp. discontinued).	
Ether:		
m/2	Constant glow 4 minutes	<5 minutes.
m/4	Constant glow 30 minutes; becomes brighter, then fainter.	<5 minutes.
m/8	Constant glow 90 minutes	<30 minutes.
m/16	Normal response >1 day (exp. discontinued).	
m		
Ethyl alcohol:		
2m	Constant glow 1 minute	
1.5m	Constant glow 5 minutes	<5 minutes.
m	Constant glow 30 minutes	<10 minutes.
m/2 to m/4	Normal response >14 hours (exp. discontinued).	
Butyl alcohol:		
m/2		
m/4	Constant glow 1 minute	
m/8	Constant glow 5 minutes	
m/16 to m/32	Constant glow 1½ hours (faint)	<20 minutes.
	Normal response >14 hours (exp. discontinued).	
Chloretone:		
Saturated	No response	
1/2 saturated	Constant glow 4 minutes	Stops immediately
1/4 to 1/8 saturated	Constant glow 15 minutes (faint)	<10 minutes.
1/16 to 1/32 saturated	Normal response >24 hours (exp. discontinued).	
Thymol:		
Saturated to 1/4 saturated.	Constant glow 1½ to 2 minutes	
1/8 saturated	Constant glow 6 minutes	
1/16 saturated	Constant glow <1 hour, normal response 4 minutes at start (poor).[1]	<15 minutes.
1/32 saturated	Normal response <18 hours (poor)	
1/64 to 1/128 saturated	Normal response >5 days (exp. discontinued.	

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[1] The normal response at the start is doubtless due to the delay in taking effect on certain of the animals,

similar experiment was tried with animals subjected to m/8 ether for 10 minutes with the same result. In these experiments the cell-membranes were destroyed, allowing oxygen to enter the cell directly, and the effect was different for the anesthetized cells and the control. The anesthetic must therefore act, not on the cell-membrane by preventing oxygen from entering the cell, but directly upon the cell interior, probably attacking the mechanism of the utilization of oxygen in the cell.

A criticism of the foregoing experiments might be offered—that, owing to the length of time that the cells were subjected to the anesthetic, substances present in the cells might be used up and the cells be fatigued. The following experiment showed that such was not the case: Three lots of animals were put into tubes with sand, two immersed in m/8 ether and the third in sea-water. One of the ether tubes was very slightly agitated almost immediately and did not increase in brightness as normal animals do on slight agitation, showing that the animals were narcotized. The other ether tube was shaken vigorously at once so as to break up the animals, and it became slightly brighter. The control tube with sea-water, when also shaken vigorously at the same time, became markedly brighter, showing a striking contrast to the other two tubes. In this experiment there was not sufficient time for any substances in the cells to be used up, and yet the narcotized cells gave a much weaker light than the control lot on admission of oxygen by destroying the membranes. The evidence from these experiments on *Noctiluca* would therefore argue against the membrane theory of anesthesia, since the anesthesia of light-production takes place independently of the cell-membrane; the effect of the anesthetic seems to be upon the mechanism of the oxidation process inside the cell.

SUMMARY.

1. The specific gravity of *Noctiluca* is less than that of sea-water, so that normally the animals float at the surface. Since they contain no air-bubbles or large oil-drops, their lower specific gravity must be due to a lower salt-content than that of sea-water. When placed in a mixture of 4 sea-water to 6 fresh-water, the animals sink, their salt-content being now greater than that of the surrounding medium, but later they rise to the surface, a process independent of the movement of the tentacle. They therefore absorb water until their salt-content is again less than the medium, thus reestablishing their normal relation to the surrounding medium. This water must be absorbed against the osmotic pressure of the salts of sea-water, a process contrary to physical laws. The animals can not only lessen their specific gravity, but can also increase it, as shown by the fact that they sink to the bottom of the sea on windy days. Anesthetics, acids and alkalies, KCN, and the pure salts of sea-water do not interfere with this regu-

latory mechanism, except when they cause irreversible changes and death; dead noctilucas always sink to the bottom.

2. Light-production in *Noctiluca* normally occurs only on stimulation of any kind, and is a momentary bright flash. The only other light-response is a steady glow occurring before death and under many experimental conditions—*e. g.*, diluting with fresh-water, addition of acids and alkalies, KCN, cold (5° to 0° C.), heat (43° to 49° C.), anesthetics, and a constant galvanic electric current.

3. In very dilute sea-water (3 sea-water to 7 fresh-water down to pure fresh-water) the animals give a constant glow for a few minutes and then die. If the sea-water is diluted with isotonic cane-sugar instead of water, a normal response is given even with 1 sea-water to 9 cane-sugar. But in isotonic cane-sugar alone a normal response is given for only a short period, followed by a steady glow and death, showing that the animals must be bathed by a medium of minimal salt-content as well as of definite osmotic pressure.

4. The normal light-response of *Noctiluca* is dependent on the balanced salts of sea-water. Isotonic Na, K, Ca, and Mg are all toxic if alone in solution.

5. n/2000 HCl and n/4000 benzoic acid in neutral artificial sea-water cause a steady glow for 20 minutes; n/2000 NH_4OH and n/500 NaOH give a steady glow for 6 and 30 minutes respectively. In NaOH the luminescence stops *before* the penetration of the alkali, and in NH_4OH *after* the penetration, as indicated by the color-change of animals stained in neutral red.

6. KCN has little effect on light-production in relatively strong concentrations. Animals in n/250 KCN respond normally for 30 minutes and then give a steady glow for 35 minutes.

7. Oxygen is necessary for light-production. Animals deprived of oxygen immediately glow on the admission of oxygen even without stimulation.

8. When the temperature is gradually raised, a steady glow appears at 42° C. and stops at 48°, when the animals die. When the temperature is lowered a steady glow appears at 5° and continues to 0°. The animals will recover on warming if kept for only a few minutes at 0°.

9. Centrifuging has no effect on the luminous response, although the protoplasm is thrown to whatever part of the cell happens to be away from the axis of the centrifuge. On standing, the protoplasm returns to its normal position under the tentacle.

10. With a constant galvanic current noctilucas flash at the make, stay glowing during the passage of the current, and stop glowing at the break with no flash. Animals will respond normally to mechanical stimulation during the passage of the current. The luminescence is uniform throughout the organisms, no polar effects being observed. Induced shocks call forth the usual flash caused by stimulation. The animals fatigue readily with interrupted induced shocks.

11. Noctilucas injured by puncturing with a needle or by a strong induced current respond to mechanical or electrical stimulation. If completely broken to pieces by pressing through cheese-cloth, the filtrate gives a constant glow, but there is no flashing on stimulation.

12. The luminescence is traceable to points of light coming from granules (photogenin) in the protoplasm. No photophelein could be demonstrated.

13. Noctilucas may be anesthetized by m/8 ether, 1/3 saturated chloroform, 1/16 saturated thymol, 1/8 saturated chloretone, m ethyl and m/8 butyl alcohol, so that they fail to give a flash on stimulation, but they always give a very faint glow; this disappears and the normal response returns on removing the anesthetic. Light-production is dependent on the consumption of oxygen. Whether the anesthesia of luminous cells is due to the fact that oxygen can not pass through the membrane, or to the fact that it can not be used, was tested by an experiment in which the cell-substance of anesthetized cells was permitted to come into contact with dissolved oxygen. Narcotized cells were broken up by shaking with sand and it was found that they produced only a faint light, whereas normal cells so treated became very brilliant. The anesthetic must therefore attack the mechanism of the utilization of oxygen in the cell, and not the permeability of the cell-membrane for oxygen.

LITERATURE LIST.

Dubois, R. 1913, 1914. Ann. de la Soc. Linn. de Lyons, vol. 60, 61.
Harvey, E. N. 1914. Carnegie Inst. Wash. Pub. No. 183, p. 131.
———. 1915. Biol. Bull. 29, p. 308.
———. 1917. Amer. Jour. Physiol., 42, p. 318.
Höber, R. 1907. Arch. f. die ges. Physiol., 120, p. 492.
Krukenberg, F. C. W. 1887. Vergl. physiol. Studien, Heidelberg, II ser., pt. 4, p. 77.
Lillie, R. 1909 to 1916. Amer. Jour. Physiol., vol. 24 *et seq.*
———. 1916. Biol. Bull. 30, p. 311.
Loeb, J., and H. Wasteneys. 1913. Jour. Biol. Chem., 14, p. 517.
Mansfeld, G. 1909. Arch. f. die ges. Physiol., 129, p. 69.
Massart, J. 1893. Bull. Sc. de la France et de la Belgique, 25, p. 59.
Mathews, A. P. 1914. Intern. Zeit. f. Physik.-Chem. Biol., 1, p. 433.
Meyer, H. 1899. Arch. f. Exp. Path. u. Pharm., 42, p. 109.
———. 1901. *Id.*, 46, p. 338.
Osterhout, W. J. V. 1913. Science, 37, p. 111.
Overton, E. 1901. Studien über die Narcose.
Quatrefages, A. de. 1850. Ann. de Sc. Nat., III ser., Zool., 14, pp. 226 and 236.
Spaeth, R. A. 1916. Amer. Jour. Physiol., 41, p. 577.
Traube, J. 1913. Arch. f. die ges. Physiol., 153, p. 276.
———. 1915. *Id.*, 160, p. 501.
Verworn, M. 1913. Die Narcose.
Warburg, O. 1910. Zeit. f. Physiol. Chem., 66, p. 305.
———. 1911. München. med. Wochenschrift, 58, p. 289.
——— and Wiesel. 1912. Arch. f. ges. Physiol., 144, p. 472.

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*25. _Endothelium and wandering connective tissue cells as seat of origin of hemoglobin._ J. H. GLOBUS, Cornell Medical School, New York City.

In the course of an investigation of the blood elements in a large number of invertebrates, the writer selected three species of annelides as best adapted for the study of the origin of hemoglobin. The intimate relationship of this respiratory pigment with erythrocytes had lead many investigators to believe that the solution of the problem of the origin and differentiation of red blood corpuscles would also uncover the ultimate source of origin of hemoglobin. This, perhaps, holds true of vertebrate blood, but in invertebrates and, especially, in annelides it is possible to find species with hemoglobin circulating free in the blood plasma. Here the way is open to trace the respiratory pigment to the group of cells, tissue or organ responsible for its formation.

The three annelides studied show wide variations in the organization of their vascular system, while they retain great similarity in the rest of their internal structures. Arenicula Cristata a marine annelide, has a very highly developed vascular system, with pulsating heart, numerous large and small blood vessels, forming a net-work about the gut and sending in branches into the body wall. Diopatra dibrahchiata, another marine annelide, shows a less complex vascular system, the vessels being arranged mainly about the gut and forming by their smaller branches a plexus. In Rhyncobolus, on the other hand, we find only a rudimentary ventral vessel, the blood fluid occilating in the hæmocoele.

In Arenicula one finds in the intermuscular connective tissue, a large number of giant cells loaded with brownish rounded pigment granules. These cells when fixed are found to be arrested in various irregular forms, indicating amoeboid movements. Their progress seemed to be in the direction of blood vessels. These blood vessels are surrounded by similar large pigment cells which seem to form their limiting wall, as no other tissue, fibrous or muscular is there discernible. Some of these cells are arrested by the killing fluid in the process of emptying their granular contents into the lumen of the blood vessel. Their granules when stained with iron-detecting reagents indicate a rich iron content. They also react readily to Eosin and other acid dyes in a way similar to the hemoglobin containing plasma and erythrocytes in vertebrates. Other staining reactions indicate that (in some stages) these granules are modified nuclear derivatives. In some cells there are discernible three types of granules showing a transition from a nuclear to a plasma reaction, with the indifferent stage of brownish granules intervening. Thus, it is reasonable to believe that these cells are concerned with the manufacture of the respiratory pigment.

Similar conditions exist in Diopatra, with the only difference that the wandering mesenchymal cells are not as numerous, and that the endothelial cells are not as large.

It is interesting to note that while in the above two species which have rather highly developed vascular channels, the hemoglobin is held in solution in the plasma, and can be traced to the cells lining the walls of the blood vessels in Rhyncobolus we have only a rudimentary vessel, and here the hemoglobin is incorporated in nucleated red blood corpuscles. Thus the origin of hemoglobin in this species is obscured and its development must be worked out along the lines of origin of the hematids. There is, however, sufficient ground to believe that these hematids are derived from the mesodermal layer of the wall of the gut.

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THE RELATIONSHIPS AND HISTOGENESIS OF THYMUS-LIKE STRUCTURES IN AMMOCOETES[1]

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THREE TEXT FIGURES AND FOUR PLATES

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INTRODUCTION

The question of the source and mode of development of the component structures of the thymus is one of the most difficult problems in anatomy Although a vast literature has accumulated during the last fifty years some of the most fundamental phases of the problem still remain unsolved.

The thymus has been described in every group of vertebrate animals from the elasmobranchs up to and including man. The involvement of gill pouches in the formation of the gland has been established in almost all of the investigated forms. There is still disagreement on the purely epithelial origin of the reticulum and Hassal's corpuscles, and the cardinal problem, the source and nature of the development of the small thymus cells, has not been definitely settled up to the present time. Three fundamentally different views, each with its coterie of supporters, are held regarding the source of these cells. A large number of investigators believe that the small thymus cells are true lymphocytes which are formed from the epithelium by a process of transformation. An equally large number believe that these cells represent true lymphocytes which have wandered into the epithelial anlage from the mesoderm. A remaining smaller group of investigators believe that the small thymus cells have an epithelial origin and are different from true lymphocytes.

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[1] This thesis has been accepted by the Graduate School of New York University, in partial fulfillment of the requirements for the degree of Doctor of Science.

Even in the most primitive animals in which the thymus has been established, the elasmobranchs, the formation of the small thymus cells does not occur until mesodermal tissue is present in the epithelial anlage. The source of a new type of cell which forms in a mixed tissue would not be difficult to determine if the two tissues entering into the formation had different morphological characters and retained them. The methods employed up to the present time have not shown sufficient morphological differences in the mesodermal and endodermal cells present in the thymus anlage to establish the source of the small thymus cells.

The sudden appearance of the thymus as a well defined structure in the elasmobranchs, together with the probability of finding a solution to the question of the source of the small thymus cells, has stimulated a number of investigators to search for a homologous structure in the more primitive types of chordate animals. While the search has been a fruitless one in the ascidians and amphioxus, various structures have been described for a thymus in the cyclostomes. The evidence offered in these descriptions has not been sufficient to establish the organ in this group of animals. The failure to find the thymus or its homologue in the cyclostomes especially in the Petromyzontes, may be attributed largely to the peculiar nature of the branchial region in this primitive group of animals.

The bearing on the interpretation of thymus histogenesis in higher animals suggested by the development of the organ in a primitive type, led the author to undertake a systematic study of the branchial region of the petromyzon larva. The time and work which have been given to this study have, I believe, been amply repaid in the results obtained. Thymus-like placodes have not only been located in the position which makes them homologous with the thymus placodes of the elasmobranchs, but the placodes have also been found in a more primitive condition than they have been shown to exist in any other animal.

LITERATURE

The search for a thymus in the most primitive chordate animals has been undertaken by a number of investigators. Up to the present time the organ has not been established in any of these lower forms. Willey ('94) suggests that the tongue-bars occurring in the gill-slits of amphioxus represents the thymus gland. The position of these structures is apparently the only basis for this suggestion. Their gelatinous structure, however, would offset any argument that they were homologous with the thymus placodes of fishes. Stannius ('84) credits the discovery of the thymus in the myxinoids to Johannes Müller. Later investigators, however, have been unable to verify this discovery. Stockard ('07) in his study of the thyreoid in Bdellostoma Stouti was unable to find a thymus in this form. M. Schultze ('56) described a tortuous sac in the ventral wall of the branchial cavity of Petromyzon planeri which he thought represented a thymus. Schneider ('79) showed that a part of this structure disappears in the development of the animal while the remaining part changes into a group of follicles which represent the thyreoid.

Schaffer ('94) described structures in the lateral branchial wall of a 51 mm. larva of Petromyzon planeri, which he thought represented thymus anlagen. He found in all twenty-eight anlagen, seven pairs on each side which consisted of ventral and dorsal portions. These anlagen were connected with the epithelium of the branchial vestibules.

The minute structure of these buds is summed up in the following:

> Was den feineren Bau dieser Knospen anlangt, so gestattet mir die mangelhafte histologische Conservierung (Alkohol) vorläufig nur folgende Bemerkungen zu machen: Von der kapsel dringen feine Bindegewebsbälkchen in das Innere ein, welche ein spärliches, reticulumartiges Stutzgerüst für die zelligen Elemente bilden. Diese selbst sind kleine Rundzellen von lymphoiden Aussehen, kaum wahrnehmbarem Protoplasma, stark färbbarem Kern und Kerngerüst. Zwischen denselben finden sich ausserdem grössere, blasse Kerne mit deutlicher Kernmembran, und Kernkörperchen, welche dem Stützgewebe anzugehören scheinen und rothe Blutkörperchen, von denen ich nicht sagen kann, ob sie frei zwischen den lymphoiden Zellen liegen oder eingeschlossen in Capillaren.
>
> Die lymphoiden Zellen sind wahrscheinlich Abkömmlinge des Kiemenepithels, wie ihr director Übergang in das letztere vermuten lässt.

In a subsequent paper Schaffer ('06) withdrew his previou interpretation and said that he did not believe these structure represent thymus anlagen.

Giacomini ('00, 1 and 2) believed that "the lymphoid organ in the basalar region of the gill lamellae (in ammocoetes) might fulfill an analogous function to the thymus in the fishes."

Castellaneta ('13) describes the structures which Schaffer found, but insists that these structures correspond to lymphoid organs in general and suggests the name 'lymphoid formations' for them.

He further calls attention to the fact that on the one side these lymphoid formations are in contact with the peribranchial vessels and on the other with the epithelium of the branchial sac. He does not consider these structures as thymus anlagen insofar that there is not a reciprocal penetration of epithelial and lymphatic elements which should occur in a thymus. Castellaneta calls attention to the abundance of lymphoid cells in the general branchial region. He suggests that these special lymphoid accumulations of Schaffer may represent a primitive condition of the thymus in which the epithelium participates only to the extent of attracting the lymphoid cells.

These lymphocyte accumulations do not occur in the part of the branchial cavity which would make them homologous with the thymus placodes in the fishes. While lymphocytes are present in these situations the evidence brought out in connection with them is not sufficient to establish their origin or the cause of their presence in these places.

A contribution on the ganoid thymus (Lepisosteus, Amia) has been submitted by Ankarsvärd and Hammar ('13). They found the organ a purely endodermal, unsegmented structure in a medial position in the dorso-caudal part of the epibranchial region. "It nevertheless has a segmented origin and the epibranchial unsegmented thymus structure represents a secondary alteration from the branchial segments." In the older developmental stages there is a rich infiltration of lymphocytes into the sub-thymic and perivascular connective tissue which stands out in striking contrast to the conditions in an earlier stage. The authors discuss the question whether this condition represents an immigration into the thymus or an emigration from it They believe that the cells have migrated from the placode and represent the beginning of an accidental involution. A lobulization of the organ does not occur. In the adult Lepisosteus the thymus is strongly involuted.

The nature of the thymus in the ganoids as described by Ankarsvärd and Hammar is so suggestive of the conditions I have found in the ammocoetes that it appears to me to represent but a very small advance beyond a primitive form in the phylogenetic development of the organ. The origin of the lymphocytes which were supposed to originally migrate into the epithelial anlage in the Lepisosteus does not appear to have been especially determined by the authors.

MATERIAL AND METHODS

The material which is the basis for this work was generously supplied to me by Prof. Simon H. Gage. It consists of a series of specimens ranging from the segmentation sphere up to and including a transforming larva and the adult These specimens undoubtedly represent two species. Petromyzon marinus unicolor and Lampetra wilderi (the lake and brook lamprey of central New York).

A number of the 5 mm specimens in my collection were kindly given to me by Prof C. R. Stockard. They were collected in Naples.

I wish to take this opportunity to express my thanks and appreciation to Professor Gage and Professor Stockard for this valuable material.

The specimens were fixed in various fixing fluids: Zenker, formol: Gilson's, Bouin's, picro-acetic, corrosive-absolute alcohol. After being imbedded in paraffin, transverse and frontal sections were cut from 4 micra to 15 micra in thickness. The sections were stained by various staining methods; methylene blue and eosin, Weigert's haematoxylin and eosin. Giemsa's eosin-azur, and haematoxylin (Delafield's) and eosin. The best differentiation was obtained by the use of the ordinary haematoxylin and eosin method.

A paraffined blotting paper model[2] of a part of the branchial region of a petromyzon larva prepared by Mr. Warburton was used in this study. A clay model of a part of the branchial region was also prepared to facilitate the study of the arrangement of the ciliated bands in the pharynx.

COMPARATIVE ANATOMY OF THE AMMOCOETE PHARYNX

The phylogenetic position of the lamprey is still a matter of speculation. Various hypothesis have been advanced in regard to its position. Some place this animal between the amphioxus and the elasmobranchs, others claim that it represents a degenerate type. However, a comparative study of the branchial region of the lamprey larva with the same region in ascidians and amphioxus on the one hand, and with the elasmobranchs on the other, suggests that the branchial region of the lamprey larva represents a transitional stage between the amphioxus and the elasmobranch types.

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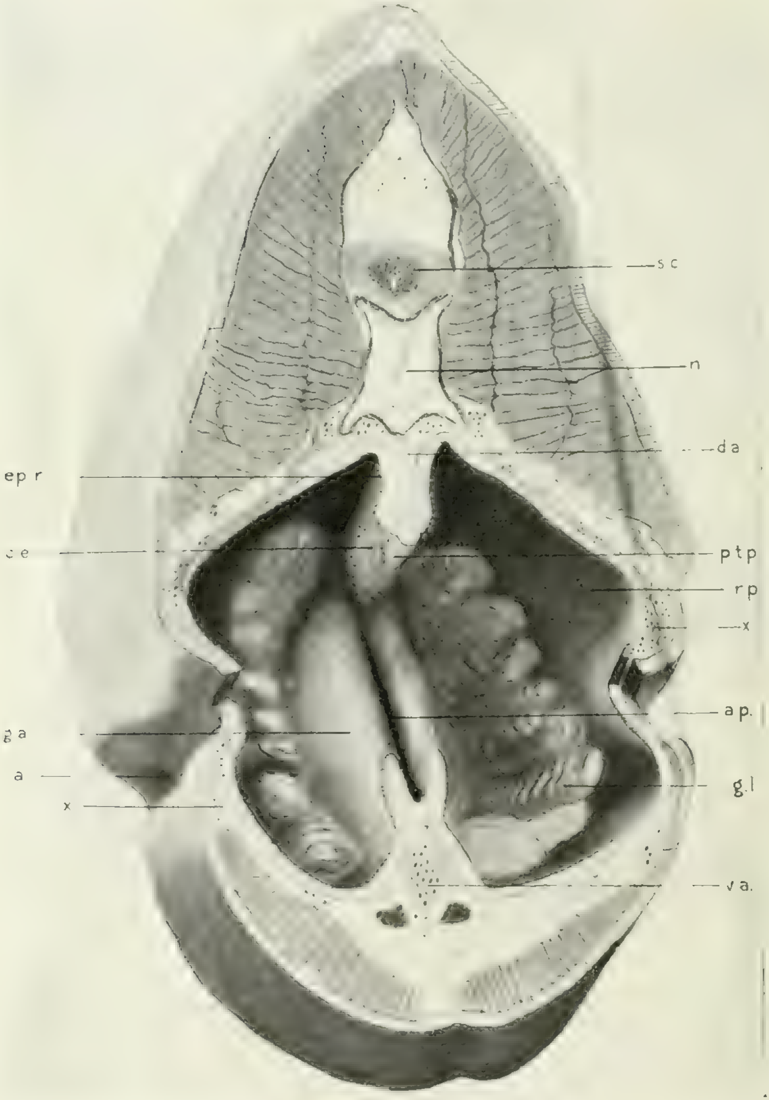
[2] A description of the method of reconstruction referred to above may be of interest to workers. This method is a modification of the late Mrs. Gage's blotting paper method. Sheets of blotting paper are dipped in melted paraffin and dried. The drawings are transferred to the paraffined paper by the usual methods when wax plates are used. The cutting is also done in the same way, the knife used, however, must have a thin but strong blade. In stacking the sections bank pins were used to hold the sections together. Small screws were alsq used occasionally to give firmness. When the stacking has been completed the sections may be smoothed down by means of any rounded instrument. A hot iron may also be used to cement the sections together. To give the best stability the complete model may be immersed in hot paraffin a few minutes. Models made in this way have a great firmness and are admirably efficient for class room use where a great deal of handling is necessary.

A few comparisons between the pharyngeal region of the ammocoete and lower and higher forms may be found in the literature. Dohrn ('84, '85) discussed the homology of the thyreoid of ammocoetes with the endostyle of ascidians and the hypobranchial ridge in amphioxus and the circumoral ciliated ring in the ammocoetes with that of the ascidians. Cunningham ('87) verifies the homologies Dohrn pointed out. Shipley ('87) calls attention to the homology of the dorsal ciliated ridge in ammocoetes and the dorsal lamellae of ascidians and the epipharyngeal groove of amphioxus.

The following considerations are based on my studies and include besides the homologies just quoted a comparison of the gills in these primitive animals:

The large branchial cavity with its medial gill arches of the lamprey larva (text fig. 1) is very suggestive of the conditions in the ascidians and amphioxus. In the ascidians there is a central pharynx surrounded by a peribranchial cavity. The two cavities communicate by means of numerous small pores, the stigmata. It is an unsettled problem whether the peribranchial cavity is derived from ectoderm or endoderm. In amphioxus there is a central pharynx which is partially surrounded by an atrium (peribranchial cavity). In this form the two cavities communicate by means of definite gill slits. The atrium of amphioxus is developed from ectoderm. In both forms there is a ventral endostyle and a structure homologous with the epipharyngeal ridge. The branchial cavity of the ammocoetes corresponds to a fusion of the two separate cavities in the ascidians and amphioxus. The primitive characters of these separate cavities, however, are still present. The central portion, that is the part bounded laterally by the gill arches (fig. 1, *a.p.*), corresponds to the pharynx of the ascidians and amphioxus.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It differs from the conditions in amphioxus in that the gill clefts are very much wider. It is probable that the ammocoete gill cleft represents the fusion of two or more gill clefts of amphioxus. There is an indication of such a fusion in the formation of the tongue-bar or secondary gill bar of amphioxus. The larger respiratory part of the ammocoete pharynx (text fig. 1, *r.p.*) corresponds to the peribranchial cavity of ascidians and amphioxus. The entire branchial cavity of the petromyzon larva is entodermal in origin. The ammocoete thus represents a phylogenetic stage in which the respiratory cavity, originally of ectodermal origin, is derived from the endoderm as it is in most higher animals.

The elasmobranch pharynx, it seems to me, represents an advanced stage of a modification which is already indicated in the ammocoete. This modification consists of a lateral migration of the dorsal and ventral attachments of the gill arches, resulting in a lateral enlargement of the central portion of the pharyngeal cavity and a consequent reduction of the respiratory part. This lateral migration is indicated by the dorsal attachments of the second pair of gill arches in the lamprey larva.

Attention may also be called to the fact that the primitive elongated character of the pharynx in the ammocoete tends to obscure its relation to the elasmobranch pharynx, in which the length has been reduced with a consequent reduction of the number and size of the gill slits.

It is necessary to determine the character of the gills in ammocoetes in so far that it has been established that the thymus in all higher forms has a more or less definite relationship to the gill pouches and gill arches.

Text fig. 1 Model of a segment of the branchial region of a 15 mm. lamprey larva. Cephalic aspect. The model shows the relationship of the primitive thymus placodes to the epipharyngeal ridge and the ciliated epithelium, as well as the relationship of the epipharyngeal ridge to the gill arches and general branchial cavity. *a.*, atropore; *a.p.*, alimentary pharynx; *c.e.*, ciliated epithelium; *d.a.*, dorsal aorta; *ep.r.*, epipharyngeal ridge; *g.a.*, gill arch; *g.l.*, gill lamellae; *n.*, notochord; *p.t.p.*, primitive thymus placode; *r.p.*, respiratory pharynx; *s.c.*, spinal cord; *v.a.*, ventral aorta; *x.*, position of accumulations of lymphocytes in lateral branchial walls.

Dohrn ('84) made the statement:

> the great difference between the Selachian, Teleost and Ganoid branchial apparatus and that of the petromyzon consists therein that the gill septa and lamellae (Kiemen-blätter und -blättchen) of the former are directed outward while in the latter they are directed inward.

He further states that this arrangement in the petromyzon exists from the beginning. This interpretation of the gills of petromyzon has been accepted in some of the textbooks on Comparative Anatomy of Vertebrates The basis for this interpretation is undoubtedly found in the position of the cartilaginous gill bars, which form a complicated branchial basket in the pharyngeal wall The branchial artery, however, is situated in the medial gill arch. From this medial gill arch the gill septum extends caudo-laterally to its attachment in the lateral wall. The gill lamellae are situated on the anterior and posterior walls of the septum. The picture of a frontal section of the gills in the ammocoetes is so much like the picture of a similar section of the elasmobranch gills that it is difficult to consider them as directed in opposite directions. The question resolves itself into a choice between the cartilaginous branchial bars and the branchial aortic arches as a basis of interpretation. It is evident that the branchial basket of petromyzon is a special modification meeting the requirements of a specialized mode of breathing due to the life habits of the adult. The position of the cartilaginous gill bars must then be considered the result of a migration from a more medial position. Moreover, the presence of the ciliated bands in the medial gill arches point to a direct phylogenetic relationship to the gill arches of amphioxus. If we consider the gills of the ammocoete as directed inward it would be necessary to consider as the gill arch, the part of the respiratory portion of the lateral branchial wall to which the gill lamella is attached. This would be contrary to the arrangement of the gills in all other chordate animals.

ORGANOGENESIS OF THE PHARYNX IN THE AMMOCOETES

A complete detailed description of the development of the branchial region in the lamprey larva is apparently not to be found in the literature. Separate structures and the condition in a single or in a limited number of developmental stages, however, have been described by various investigators. These descriptions have been accurate with the exception of minor details, but having been limited to a single stage in most cases they do not include the changes which occur with the growth of the larva. There are consequently contradictory statements in the literature on the pharynx of the ammocoetes and especially in the part dealing with the ciliated grooves and bands. Further, the formation of structures which I interpret as primitive thymus placodes is closely linked with the changes which occur in the ciliated bands in the branchial lining.

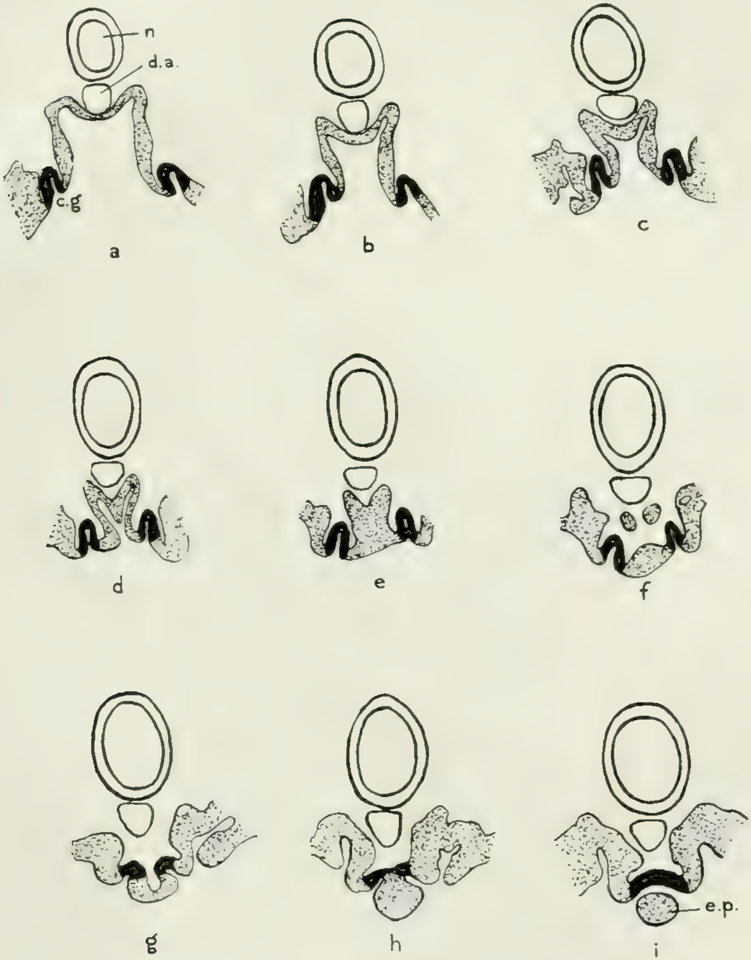
The following descriptions are based entirely upon my own material:

The transformations in the early larvae are very rapid so that in 6 and 7 mm. larvae gill lamellae have formed on the branchial septa, the pouches open to the outside, and the epithelium is represented by more than one layer. A system of ciliated epithelial grooves and bands are present in this stage of development of the pharynx. They form a connected system which may be looked upon as beginning in two rather deep diverticula in the caudal walls of the first pair of gill pouches. From each diverticulum two ciliated grooves originate, one passing ventro-caudally, the other dorso-caudally. These will be designated the ventral and doısal grooves respectively.

The two ventral grooves converge in a caudal direction as far as the third pair of gill pouches where they come to lie close together and parallel to each other near the median line. Between the two grooves is a median ridge of non-ciliated epithelium which disappears in the fourth pouch where the two ciliated grooves fuse to form a single one. A tubular divericulum passes from the ventral groove into the thyreoid a short distance caudad of the point where the grooves fuse. A second diverticulum connects the thyreoid with the fifth pouch. In the seventh pouch the ciliated groove ends. It is directly continued by non-ciliated epithelium which, in a few sections caudad, becomes a ridge. The ciliated groove is a continuous groove from the fourth to the seventh pouches. The ridge which begins in the seventh pouch gradually becomes high and stalked in the eighth pouch. The median columnar epithelium becomes invaginated and is directly continuous into the floor of the oesophagus. Surrounding the junction it seems to me there is evidence of a vestigial eighth gill arch in which an aortic arch is not present. The respiratory part of the branchial cavity extends a short distance caudad of the point of junction between the pharynx and oesophagus.

The dorsal ciliated grooves arising in the diverticula follow the course of the first aortic arch to the median dorsal line of the pharynx. They fuse at this point to form a single ciliated band which extends caudally the whole length of the branchial cavity and which is directly continuous into the roof of the oesophagus. A short distance caudad of the point of origin, this band forms a rounded ridge which extends to the seventh sac where it is converted into a groove. The aorta is lodged in the concavity of the rounded ridge. At the point where the two dorsal grooves of the first pouch fuse a tongue-like piece of non-ciliated epithelium is pinched off (text fig. 2). Schaffer apparently mistook this for ciliated epithelium and considered it the end of the fused ciliated bands.

The first pair of gill arches come together dorsally in the median line. Their ventral extremities, however, are far apart and end in the ventro-lateral part of the respiratory pharynx. Gill lamellae are present only on the caudal surface of the first gill septum. The second pair of arches are farther apart and thus they differ from the remaining caudal arches. Their dorsal attachments are in the angle between the epipharyngeal ridge and the dorso-medial part of the respiratory pharynx. Ventrally, the second gill arches are attached about midway between the mid-ventral line and the ventro-lateral angle of the respiratory pharynx. The dorsal attachments of the third and remaining pairs of gill arches is the ventro-lateral angle of the epipharyngeal ridge. Ventrally, they are attached near the ventral median line, a little to the side of the endostyle. The second pair of arches are peculiar in that they contain no cili-



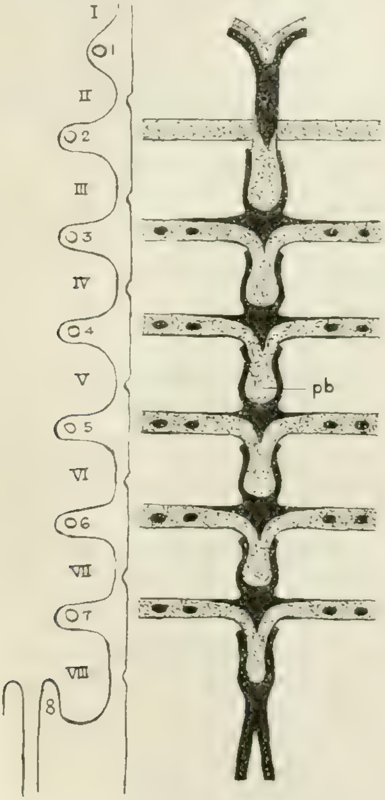
Text fig. 2 Camera lucida outline drawings to illustrate the course of the dorsal ciliated grooves (the ventral grooves are not shown) from the diverticula in the first pouch to the point where the grooves meet in the median dorsal line to form a single band of ciliated epithelium. *c.g.*, dorsal ciliated groove; *d.a.*, dorsal aorta; *e.p.*, epithelial placode; *n.*, notochord.

ated bands. The third and remaining arches have a broad ciliated band covering the medial and cephalic aspect These bands are directly continuous with the ciliated band on the epipharyngeal ridge. They have no connection with the endostyle in this stage of development and I have been unable to determine whether such a connection exists or not in younger larvae.

The moving apart of the second pair of gill arches is very suggestive of an approach to the condition in fishes where the arches are attached in the lateral part of the roof of the pharynx. Accompanying this lateral migration there is a loss of the ciliated band on the arch.

The arrangement of the ciliated bands as described above does not persist in older larvae. This undoubtedly accounts for the contradictory descriptions given by Anton Schneider ('79) and Schaffer ('95, 1 and 2) and others. In a larva 9.5 mm. in length growth and differentiation of the epithelium of the gill arches has resulted in a new arrangement of the ciliated bands. This new arrangement has gained its permanent larval condition in a 15 mm. larva.

In the older larvae the median dorsal ciliated band which represents the fused continuation of the dorsal ciliated grooves of the first arch ends in the median dorsal line between the second pair of gill arches. Immediately caudad of the dorsal attachment of the second pair of gill arches two ciliated bands appear on the ventro-lateral part of the epipharyngeal ridge. Tracing these bands in a caudal direction, they are seen to come together and fuse in the median ventral part of the ridge at the caudal end of the dorsal equivalent of the third gill pouches. From this single band a branch is given to each of the third pair of gill arches. In the median line the ciliated band ends as a pointed process in the angle between the dorsal attachments of the third pair of gill arches. This arrangement of the ciliated bands is repeated in the remaining arches and dorsal equivalents of the gill pouches (text fig. 3, and *c.c.* in text fig. 1). In the eighth pouch, however, the median ventral fused part does not give off any lateral branches corresponding to the ones given off to each gill arch in the third to the seventh arches. This is due to the nature of the dorsal attachment of what I have assumed must be the eighth vestigial gill arch. This arch, like the second, is not attached to the epipharyngeal ridge but to the angle between the ridge and the dorsal wall of the respiratory pharynx. Cilia, however, are present on the lateral side of the eighth gill arch. They undoubtedly represent the vestigial remains of a condition in which this arch had a dorsal attachment to the ridge or its equivalent. The median ciliated band



Text fig. 3 Diagram illustrating the arrangement of the ciliated bands on the epipharyngeal ridge and the gill arches in a 31 mm. larva. Ventral view. The gill arches which extend ventrally are here represented as extending laterally. The ciliated epithelium is represented in heavy black, the non-ciliated by the stippled part. Roman numbers indicate the gill pouches and Arabic numbers the gill arches. *pl.*, primitive thymus placodes.

of the eighth pouch is directly continuous into the oesophagus. It is interesting to find that the band divides into two portions within the oesophagus. These perhaps represent the branches which were given off to the eighth pair of gill arches in an ancestral form.

Patches of ciliated epithelium are also present on the medial aspect of each gill arch from the third to the seventh inclusive. These ciliated patches undoubtedly were a part of the single ciliated band on the gill arches in the younger larva. With the growth of the non-ciliated epithelium of the gill arches, these patches were cut off from the ciliated band. In the older larva the ciliated band of the gill arch does not occupy the same relative position that it did in the young larva. The ventral end is situated on the lateral side of the gill arch. When traced in a dorsal direction it is found to take a slightly spiral course so that the dorsal extremity which is continuous into the ciliated band of the epipharyngeal ridge comes to occupy a cephalomedial position. This change in the course of the ciliated band may also be looked upon as the result of the growth of the non-ciliated epithelium. The ciliated patches on the medial side of the gill arch may acquire a sensory function as Schaffer suggested.

The arrangement of the ciliated epithelium on the epipharyngeal ridge in the older larvae is also the result of the growth of the non-ciliated epithelium. In a larva between 8 and 9 mm. in length the non-ciliated epithelium of the dorso-medial part of the gill arches begins to invade the ciliated epithelium of the epipharyngeal ridge. As a result of this invasion the continuity of the ciliated band on the epipharyngeal ridge is lost. The two cords of invading epithelium from the opposing gill arches fuse in the median ventral line of the ridge. The invasion is continued in a caudal direction dividing the ciliated band into two portions which are pushed laterally. This fused portion (*pl.*, text fig. 3) becomes thicker and broader in a caudal direction and ends a short distance cephalad to the attachment of the next pair of gill arches At the caudal end of this invading epithelium the subsequent growth does not divide the ciliated epithelium further, but produced a tongue-like process which projects into the pharyngeal cavity similar to the tongue-like non-ciliated epithelial process represented in text figure 2).

The non-ciliated epithelium which has invaded the ciliated epithelium of the epipharyngeal ridge begins to show histogenetic activities in a larva between 20 and 30 mm. in length. The nuclei of the cells of these areas, or placodes, wander out into the underlying connective tissues and are transformed into lymphocyte-like cells. A study of the histogenetic processes in these areas in various developmental stages leads to the conclusion that these areas represent specialized regions of the branchial epithelium which are suggestive of primitive thymus structures.

From the foregoing description it is evident that there are in all seven placodes. The seventh and the first are smaller than the remaining ones but they take part in the histogenetic processes and are therefore to be considered true functional placodes. The placodes increase in size with the growth of the larva. In the mature larva, however, they show a depletion of cells.

When the larva undergoes metamorphosis the whole structural arrangement of the branchial-region is altered. In the single specimen of a transforming larva of my collection, the adult arrangement has been attained, so I am unable to describe the nature of this process. In this transforming specimen I have also been unable to find any remains of the epithelial placodes of the larva. Serial sections of the branchial region of an adult lamprey have also been examined but with negative results. It is evident that an involution of the placodal organ has taken place as one would expect of a thymus. This involution began in the maturing larva and was completed in the early stages of metamorphosis.

HISTOGENESIS

Early development of the pharyngeal wall in Petromyzon marinus unicolor, Lampetra wilderi

The search for a thymus in the lamprey larva has revealed an unusual accumulation of lymphocytes in the lateral walls of the branchial cavity. These accumulations were first observed by

Schaffer ('94) and later described more in detail by Giacomini ('00, 1 and 2) and Castellaneta ('13). The origin of these lymphocytes as well as the cause for their accumulation in these places has not been determined and consequently constitutes a problem to be solved in the consideration of a possible thymus structure in this animal. The descriptions in the literature have been limited to single stages of development and are consequently incomplete. The following descriptions are based on my own material and includes the essential developmental stages:

The branchial cavity of a 5 mm. larva represents a very simple condition. The gill pouches are present as wide evaginations extending to the ectoderm in the mid-lateral plane leaving the gills as simple projections of the lateral endodermal walls. Loose mesenchyma cells fill up the space within the gill. The aortic arches are forming near the free medial border of the gills, but the vascularization of the body of the gill has not begun as yet. Although the larva contains a great quantity of yolk in this stage the branchial region is quite free from it.

The epithelium lining the branchial cavity including the gills consists of a single layer of columnar cells. Dorsal and ventral to the mid-lateral plane of each gill pouch and corresponding to the position of the future lymphoid accumulations of Schaffer the endoderm shows a slight thickening (fig. 2). In these places which may be called placodes the cells have lost their columnar shape and their outlines have more or less disappeared. The area appears to be taking on a syncytial character in which the nuclei do not have any definite grouping.

Marked changes have occurred in these placodes in a 9.5 mm. larva. In general, the placodes have become enlarged both in thickness and area (fig. 3). Cell outlines are practically all obliterated. The cytoplasm is streaky in appearance suggesting a degeneration. The nuclei exhibit variations in character. Some are very dark with a large chromatin content while others are pale and contain a small amount of chromatin. Still others show amoeboid characters. I have not been able to determine whether all these nuclei are indigenous to the placode. The general epithelium of the branchial cavity has also acquired a new character in this stage. Beneath the placodes and in direct contact with them the peribranchial blood channels are forming. It is difficult to distinguish an endothelial lining of these channels in all cases.

The degeneration of the placode has progressed farther in a 15 mm. larva (fig. 4). In places the cytoplasm has taken the stain very faintly. Scattered about in the placode are streaks of cytoplasm which are very deeply stained. Vacuoles are also present. The nuclei appear to be fewer in number than in the earlier stages. They appear more constant in their general appearance and chromatin content. The amoeboid character of the nuclei has also become more prominent. Some nuclei have taken up positions at the surface of the placode and the cytoplasm appears to be cutting off a layer of flattened cell (fig. 4, *s.l.*). The formation of a layer of flat cells at the surface of the general epithelium was begun in a much earlier stage of development.

The changes which occur in these placodes in older larvae approximate the character represented in the 63 mm. larva (fig. 1). It is a significant fact that cells in mitosis have not been seen in any stage of the development of these placodes. Furthermore, patches of epithelium giving the appearance of a degeneration are present in various other parts of the branchial lining, especially at the lateral attachments of the gill septa. Lymphocytes are present in the placodes in the older larvae, but they are also present in the general branchial epithelium. They do, however, occur in greater numbers at these placodes.

From these brief descriptions it is apparent that these placodes do not represent active anlagen of a future structure. Their development and structure do not suggest anything which might indicate their significance.

The lymphocyte accumulations in relation to the above described placodes are contained within vascular channels. These vascular channels contain red blood cells, and as has been shown by Mozejko ('10) and others they are in communication with similar channels within the body of the gill as well as with the definitive blood vessels of the pharynx.

Lymphocytes begin to make their first appearance in the blood in larvae of about 9 mm. length. They increase in number with the growth of the larvae, but chiefly remain outside of the main blood vessels. They are especially abundant in the perivascular spaces of the gill arches.

The accumulations of lymphocytes in the lateral branchial walls are foreign to these situations so far as their origin is concerned. Furthermore, the epithelial placodes in these situations together with the lymphoid accumulations do not exhibit the characters which are essential in either a well-established or rudimentary thymus. I can offer no suggestion in regard to any special significance of these accumulations. They appear to be merely a part of a rich accumulation of lymphocytes in the connective tissue spaces of the branchial region. It is probable that the apparently degenerating placodes play a rôle of attraction for lymphocytes.

Epipharyngeal placodes

The placodes in the epipharyngeal ridge are present in an undifferentiated condition in a 15 mm. larva. They form distinct masses of cells in the mid-ventral part of the epipharyngeal ridge between the ciliated bands. They are very nearly circular in outline in a transverse section, producing a bulging into the interior of the ridge (figs. 5 and 6). A loose mesenchymatous tissue caps the dorsal surface of the placodes. Red blood cells are occasionally present in the spaces of the mesenchymal tissue. These spaces are apparently in communication with the dorsal aorta by means of minute apertures and are directly continuous with similar spaces in the connective tissue of the gill arches. Through the spaces of the gill arches a communication is also made with the peribranchial sinuses and the perivascular spaces in the gills.

Within the placode the cells are in an active state of proliferation. The nuclei of the resting cells are rather clear structures with the chromatin generally collected into small lumps situated next to the nuclear membrane. The nuclei are smaller than those of the ciliated epithelial cells. Cell outlines are more or less distinct in the placodes. A peculiar type of vacuolization is in progress in some of the cells in which the complete cell becomes vacuolated leaving the protoplasmic remains as free bodies within the hollow cell. The protoplasmic bodies in these cases are small lumps of nucleated protoplasm in which the nuclear material generally stains an intense black and the cytoplasm a light red. These protoplasmic bodies are not limited to the epithelial placodes, but may be found everywhere in the branchial epithelium, especially in the 15 mm. larva.

A layer of flat cells clothes the surface of the placode This layer is not distinct in every section and may easily be overlooked. At the connective tissue border of the placode a basement membrane sharply marks off the epithelium from the mesoderm (*b.m.*, fig. 5). Lymphocytes have not been found in the placodes in a 15 mm. larva although they are present in the blood. They may be seen, however, in the general branchial epithelium and also in the ciliated epithelium (*lm.*, fig. 6).

The placodes have increased considerably in size in a 31 mm. larva. The mesenchymatous tissue which was present above the placode in the 15 mm. stage has changed to connective tissue (fig. 7). Large spaces containing various kinds of blood cells are present in this connective tissue. Larger and smaller nuclei may be seen in the walls of these spaces. The smaller undoubtedly represent the nuclei of endothelial cells. The larger, however, are apparently derived from the placode and are in a stage of migration into the vascular spaces. It is doubtful whether these spaces should be considered true blood channels. While red blood cells are quite abundant in the spaces in this stage of development they are practically absent in them in the full grown larva. It is probable that they represent a primitive type of lymph vessels, as has been suggested by various authors. The connective tissue is of the fibrous variety in which the individual fibers are quite slender. The fibers interlace to form a loose mesh work. Figure 7 represents a transverse section through the cephalic part of a placode with its neighboring connective tissue and the ciliated epithelium of one side. In such a region the nuclear elements are very scarce in the connective tissue when compared to the region above the central part of a placode. The basement membrane which is present only on the right-hand side in the section illustrated in figure 7 (*b.m.*) bridges across the entire placode a few sections cephalad of the one illustrated. In the central part there is no line of demarcation between the placode and the connective tissue. The cytoplasm of the placode in this place is directly continuous with the connective tissue.

The cells within the placode have greatly increased in numbers in this stage of development. Near the free surface of the placode they are loosened from each other, displaying their rounded outlines distinctly. Toward the deeper part of the placode the cells become oblong in shape. Near the connective tissue border the cell outlines are lost which gives the appearance of a syncytium. The nuclei of the cells in the placode are not unlike the nuclei of the ciliated and the general branchial epithelium in their general morphological characters, except in size. They are smaller than the nuclei of the ciliated and general epithelium. The chromatin of the nuclei, for the most part, is collected into a single lump which stains a reddish-purple with the haematoxylin-eosin stain. The nuclei also change from a circular to an oblong outline from the free surface of the placode to the connective tissue border. At the place where the cytoplasm of the placode is continuous with the connective tissue, the nuclei become quite elongated, having the appearance suggestive of a migration into the connective tissue. This migratory appearance is more prominent at the central part of the placode. Figure 10 represents a part of a transverse section from the central region of the placode. The two lower nuclei marked *a* in the figure lie in the cytoplasm of the placode. All the nuclei and cells above this level are in the connective tissue and spaces above the placode. The nuclei in the connective tissue show degrees of gradual variations in morphological characters from the typical epithelial nucleus of the placode to the mature lymphocyte-like cell. Nuclei showing these degrees of variation may all be found in a single section. Figures 10 and 11 show the most obvious stages in this gradual variation. The nuclei marked *a* are typical placode epithelium nuclei apparently in a state of emigration. The nucleus marked *b* is in the connective tissue. The chromatin in this nucleus is apparently breaking up into a number of granules, a process which has proceeded farther in nucleus *c*. In nucleus *d* the chromatin granules are apparently arranging themselves on the nuclear membrane, an arrangement which has been completely attained in nucleus *e*. Nucleus *e* further shows a tendency towards acquiring a circular outline which becomes more manifest in nucleus *f*. Nucleus *f* also shows a reduction in size. Nucleus *g* (fig. 11) has a circular outline and further shows a change in the character of the protoplasm. The nucleus *h* shows a still further reduction in size, the protoplasm stains darker as does also the chromatin. The chromatin, further, forms a continuous layer at the periphery. In *i* (figs. 10 and 11) the nuclei have acquired a thin covering of cytoplasm which is not visible at all points of the nuclear surface. The cytoplasm stains a gray-blue. The nucleoplasm and the chromatin of these cells take the stain more intensely than the nucleus *h*. These cells have also become free from the connective tissue mesh work. In the cells *j* the nucleoplasm stains a deep purple. The chromatin appears to have left the nuclear membrane and is now present as granules scattered about in the nucleus. In some nuclei the chromatin granules are connected together by slender processes, in others this is apparently not the case. Still other cells show nuclei in which the chromatin is represented by a single large lump. These cells (*j*, figs. 10 and 11) represent the typical lymphocyte-like cell in this region of the 31 mm. larva. Some of these cells may be found in which the nucleoplasm stains a gray-blue (*k*, fig. 10). They are similar to the lymphocytes in older larvae and may either represent a final stage in the development of the cells, or they may represent cells foreign to this locality.

From the above described transitional conditions and from a study of the stained sections. I can draw no other conclusion than that, the nuclei of the epithelial placode transform into lymphocyte-like cells. It is a significant circumstance that the nuclei alone migrate from the placode, i.e., no cytoplasm is visible. Complete cells bearing epithelial characters may be found in the connective tissue spaces. However, I have never found them migrating from the placode while I have found migrations of the complete cell from the epithelium of other regions.

Cells in mitosis may be seen occasionally in the placodes of the 31 mm. larva. Cells in a state of amitotic division, however, are quite abundant in a 44 mm. larva, suggesting that cell-proliferation takes place chiefly by simple fission. Figure 15 shows the nucleus of a placode cell apparently in a process of simple fission. Mitotic cells are especially scarce in the connective tissue above the placode. A single instance has been found and is represented in figure 18. It is quite evident from the lack of mitotic or amitotic cells or nuclei in the connective tissue that cells or nuclei are not being formed in any significant quantities in this situation.

Transformation stages have not been found within the placode in the 31 mm. larva. Lymphocyte-like cells, however, are present in the placodes. Their presence may be accounted for by means of an immigration from the connective tissue.

The further development of the placodes is a repetition of the above-described processes except that the transformation is more rapid and begins within the placode. Figure 8 represents a part of a transverse section of the placode and the connective tissue above it in a 44 mm. larva. The illustration was drawn to the same magnification as figure 7. The nuclei in the placode are elongated and show amoeboid characters. They also appear to be in an active state of emigration. The transformation process appears to have begun in the placode in this stage. The nuclei near the connective tissue border have taken on characters which approach the characters of some of the nuclei in the connective tissue. This change is shown in the staining reaction, the condition of the chromatin, and the shape of the nuclei. The nuclei at the border show a tendency to stain blue, the chromatin takes a darker stain, and in some cases is broken up into granules, and the nuclei approach the globular shape. The transformation of the nuclei in the connective tissue appears to be of the same character as in the 31 mm. stage, but apparently more rapid. Nuclei may occasionally be found in the placode which show phagocytic properties. Figure 14 represents a placode nucleus in the act of engulfing protoplasmic bodies.

The spaces in the connective tissue in the 44 mm. larva appear to be smaller than in the 31 mm. stage. Some of them have a distinct wall while others appear like transient spaces in the connective tissue. Red blood cells are only occasionally seen in the connective tissue spaces of this stage.

The placodes in a 63 mm. larva are larger in area but thinner than in the preceding stages. The nuclei of the epithelial cells of the placode have lost their original character. The chromatin is no longer represented by a single large lump, but is present in the form of granules corresponding to the chromatin in the nuclei which had migrated into the connective tissue in the 31 mm. stage. The number of lymphocyte-like cells has increased considerably within the placode. All the stages of transformation from epithelial nucleus to the mature lymphocyte-like cell may be found within the placode in this stage of development. A basement membrane is re-forming at the connective tissue border of the placode. The 'vascular spaces' of the connective tissue are now chiefly limited to the peripheral part of the whole connective tissue within the ventral half of the epipharyngeal ridge. The mature lymphocyte-like cells are chiefly located in these channels, leaving the central connective tissue core quite free from cells. The central core consequently has a much lighter appearance. Some nuclei are present in the central core, the morphological characters of which are similar to the characters of connective tissue nuclei in other parts of the body. Other nuclei may occasionally be seen in which the characters agree with the various transformation stages of the lymphocyte-like formation shown in younger larvae.

The activities within the placodes of the full grown larva (120 mm.) have diminished and are apparently approaching a condition of cessation. The number of epithelial nuclei has been reduced considerably. Transitional stages may be found, but are quite scarce. Mature lymphocyte-like cells are also present, but not in great numbers. A definite basement membrane is now present at the connective tissue border of the placode. Figure 9 represents a portion of a transverse section of the placode and the tissue above it in a 120 mm. larva. The section is taken near the cephalic end of the placode. In such a region a peculiar formation has occurred in the connective tissue, the significance of which I am quite unable to explain. This formation consists of what appears to be red blood cells held in the meshes of the connective tissue (*x*, fig. 9). The cells have the morphological characters of the red blood cells. The cytoplasm has a decided yellow tint, while the pale nuclei have a green tint. In some cases what appears to be the nuclei have morphological characters similar to the lymphocyte-like cells. These formations are present in the periphery of the whole connective tissue. A section through the central part of the placode would show the same character that was indicated in the 63 mm. larva, that is, a central core of connective tissue in which there are no 'vascular channels' surrounded by a 'vascular area.' The tissue between the 'vascular channels' in the 120 mm. larva consists entirely of the peculiar tissue just described.

Development of lymphocytes

A brief description of the general development of lymphocytes in the petromyzon larva is here given since the nature of this formation in the advanced larvae has a direct bearing on the interpretation of the histogenetic processes in the above described placodes. My observations do not include the first appearance and development of the blood in the embryo, but begin with the development in the 5 mm. larva. The nature of the blood formation in this stage of larval development need not be described here for the reason that it occurs at a time when the placodes have not begun to form. However, in larvae ranging from 9.5 mm. in length up to the mature individual, blood cells develop from the epithelial cells of the gills, gill arches, and probably the branchial wall by a process of transformation. It is the blood formation occurring in the gills and gill arches which is of especial interest in connection with the histogenesis in the placodes. The description of this formation will be limited to the formation of lymphocytes only, in the 31 mm. larva.

The similarity of the cytoplasm of the gill epithelium to the cytoplasm of some of the blood cells was early noticed. This similarity was found to be due to an actual relationship between the two kinds of cells and thus not a mere coincidence. This relationship was demonstrated when epithelial cells were found migrating through the walls into the lumen of the blood channels in the gill. Figure 13 represents a part of the gill epithelium and a blood vessel and shows an epithelial cell beginning its migration into the vessel. Figure 12*a* shows another epithelial cell in the state of migration, almost half of the cell in this case is inside of the vessel. The cells to the left in figure 12 represent blood cells (in the vessel) in various stages of transformation. In this figure, the chief stages in the transformation of the epithelial cells to lymphocytes are represented. The lettering *a* to *h* in the figure shows the line of transition from an epithelial to the mature lymphocyte.

In this formation of lymphocytes, it is noteworthy that the entire epithelial cell migrates from the 'epithelium' and takes part in the transformation. The transformation consists of a reduction in the size of the nucleus and also in the amount of cytoplasm. The cytoplasm retains its staining qualities through these changes so that even in the mature lymphocyte a cytoplasmic ring which stains red may be seen in many instances. It is very seldom that a lymphocyte containing a cytoplasm which stains a blue or gray-blue is seen in these situations. All the transforming cells have a cytoplasm which stains red with the haematoxylin-eosin stain.

Although some of the epithelial cells in this transformation migrate directly into the blood vessels, the great majority wander into the perivascular spaces and undergo their transformation in these places. The sluggish character of the blood flow in these spaces must account for the retention of the large number of transforming and mature lymphocytes which are present in these situations. The entrance of these cells into the main blood vessels is of a slow nature.

The tall epithelial cells in the dorsal part of the epipharyngeal ridge also enter into the blood formation. Figure 16 shows a cell taken from the space in the connective tissue of the dorsal part of the epipharyngeal ridge. The nucleus has the morphological characters of the epithelial nuclei. It appears to be in a state of simple fission. The cell in figure 17 was taken from the same locality. Two nuclei are present in this cell which still show the epithelial character.

Histogenetic comparisons between cells arising from placodes and lymphocytes

In the study of the histogenetic processes in the placodes it was shown by means of various transitional stages that the epithelial cells of the placode transform into lymphocyte-like cells. Lymphocytes were shown to develop from the 'epithelial' cells of the gills and gill arches. The lymphocyte-like cells formed from the placodes do not have the same mode of development nor do the transitional forms have the same morphological characters as the lymphocytes and transitional forms developed from the gill and gill arch 'epithelium.' In the placode the nuclei alone migrate away from the original epithelial bed and the transformation occurs in the connective tissue meshwork. The complete cell migrates away from the epithelial bed in the gills and gill arches, the transformation occurs in the perivascular spaces and the blood vessels. A small amount of cytoplasm becomes visible in the placode 'lymphocyte' just before it attains its maturity. This cytoplasm stains a gray-blue. The cytoplasm of the gill and gill arch lymphocytes represent the original cytoplasm of the 'epithelial' cells and stains red. These important differences in the lymphocytes and lymphocyte-like cells occur in the same section and thus cannot be attributed to difference of technique. A lymphocyte with red cytoplasm may occasionally be found in the epipharyngeal ridge just as a 'lymphocyte' with gray-blue cytoplasm may occasionally be found in the gill region. The great majority of 'lymphocytes' in the placode region, however, contain cytoplasm which stains gray-blue. It was also pointed out above that the lymphocytes in the gill region are chiefly the type which have red cytoplasm. The presence of the lymphocytes with red cytoplasm in the placode region and the type with gray-blue cytoplasm in the gill region may be accounted for by migration from their seats of origin. They may also be brought to these situations by the flow of the blood.

On account of the morphological difference of the developing lymphocytes in the gill region and lymphocyte-like cells of the placodes, the conclusion seems justifiable that the placodes are segregated portions of the 'epithelium' representing individual organs which produce cells of a lymphocyte appearance, but differing from the lymphocytes formed in the 'epithelium' of the gills.

DISCUSSION

The data submitted in the consideration of the lymphocyte accumulations in the lateral branchial wall of the lamprey larva does not supply any evidence that these formations represent primitive thymus anlagen. Although placode-like formations are present in the lateral branchial wall, similar formations are also present in other parts of the pharyngeal epithelium.

An important component of the thymus of higher animals is a reticulum. In my study of the thymus-like placodes in the lamprey larva, I have been unable to find any undisputable evidence of a reticulum in the placode. At the connective tissue border of the placode the epithelial cytoplasm apparently has a fibrous character (fig. 10). I have been unable to determine whether this represents connective tissue or transformed epithelial cytoplasm. Judging by its appearance and position it probably represents connective tissue which has been invaded by cytoplasm from the placode. The connective tissue outside the placode plays the role of a reticulum insofar that the transformation of the epithelial nuclei occur within its meshes.

Hassal's corpuscles, or any structures comparable to them have not been found in the placodes or in the connective tissue outside of the placode.

The history of the placodes in the successive developmental stages indicates a gradual involution of the placodes. The maximum size of the placodes occurs in a larva of 50 to 60 mm. in length. From this stage of development the placodes diminish in size so that in the mature larva very few lymphocyte-like cells remain. In the transformation of the larva, Nestler ('10) maintains that the oesophagus of the adult is formed by a transformation of "the under edge of the dorsal fold in the branchial chamber" (the epipharyngeal ridge). If such a process occurs, is is only after the histogenetic activities in the placodes have ceased and consequently does not affect the status of an earlier thymic function in these placodes.

An examination of the descriptions given in the preceding pages give the impression that the primitive thymus placodes and lymphocytes are formed from an endodermal epithelium. While I am not ready at this time to supply the evidence, the changes which occur in the general branchial epithelium in the early stages of development seem to point to a general fusion of the original endoderm with the underlying mesenchyma. The character of the epithelium in the more advanced larvae has such an important bearing on the interpretation of the histogenesis of the primitive thymus cells and lymphocytes that a separate and detailed study of this process seems warranted.

In a recent article on the Development of the Human Pharynx, Kingsbury ('15) discusses the intrinsic and extrinsic factors in thymus formation and challenges the view that the thymus is a branchiomeric organ definitely located in the branchial epithelium. The basis for this interpretation

is found in the recognition that it is a structure whose appearance is determined by extrinsic factors of relation and position and not intrinsic factors located in any particular group of cells. In support of such an interpretation and giving us, I believe, a better comprehension of its morphologic significance, we have the fundamental plan of its histogenesis.

The true nature of the endodermal-mesenchymal relationship in the ammocoete pharynx has not been definitely determined. Whatever these extrinsic factors may be, they are apparently of the same nature in the thymus-like placodes and the lymphocyte-forming 'epithelium' of the branchial arches. The products of these two regions, however, are not similar and it seems to me that this dissimilarity can only be explained on the basis of an intrinsic value or specificity of the 'epithelium' of the placode.

The nature of the formation of lymphocytes and the primitive thymus placodes in the lamprey larva point to an ontogenetic relationship in the histogenesis of thymus cells and lymphocytes. The branchial region of the lamprey larva may be looked upon as possessing general lymphocyte-forming properties in which the primitive thymus placodes represent specialized regions of the general lymphocyte-forming 'epithelium.'

SUMMARY

From the evidence obtained in this investigation of the ammocoetes the following conclusions seem justified:

The placodes in the lateral branchial wall are apparently patches of degenerating epithelium and have nothing to do with a thymus structure. The collection of lymphocytes at these places are foreign to this situation so far as their origin is concerned.

The gills in the ammocoetes are homologous with and extend in the same direction as the gills in elasmobranchs.

The branchial 'epithelium' does not represent a pure endodermal epithelium. This 'epithelium' develops haemopoetic properties in the advanced larva.

'Epithelium' from the gill arches invades the ciliated epithelium of the epipharyngeal ridge and produces placodes. These placodes have a relationship to the gill arches and gill pouches which makes them homologous with the thymus placodes of elasmobranchs and are to be considered primitive thymus structures.

The lymphocyte-like cells which originate in the primitive thymus placodes have different morphological characters and have a different mode of formation than the lymphocytes which are formed in the gill arches and lamellae.

This investigation has been pursued in the laboratories of anatomy at Cornell University Medical School and Marquette University Medical School. While I hope to have established a primitive thymus structure in the ammocoetes, many of the important problems of the histogenesis of the lymphocytes and primitive thymus cells must be left undecided until more exhaustive investigations can be completed.

BIBLIOGRAPHY

Ankarsvärd, G., und Hammar, J. 1913 Zur Kenntnis der Ganoidenthymus. Zool. Jahrb. Abt. f. Anat. u. Ontog. der Tierre, Bd. 36, p. 3.

Castellaneta, V. 1913 Sulla questione del timio in 'Ammocoetes.' Monitore zool. Italiano, Anno 24, pp. 161–174.

Cunningham, J. Y. 1887 Dr. Dohrn's inquiries into the evolution of organs in the chordata. Quart. Journ. of Micr. Science, vol. 27, pp. 265–266.

Dohrn, A. 1884 Studien zur Urgeschichte des Wirbeltierkorpers. IV. Die Entwickelung und Differenzierung der Kiemenbogen der Selachier. Mitteil. a. d. Zool. Station zu Neapel, Bd. 5.

1885 Studien zur Urgeschichte des Wirebeltierkorpers; VII. Entstehung und Bedeutung der Glandula Thyreoids; VIII. Die Thyreoidea bei Petromyzon, Amphioxus und den Tunikaten, Mitteil. d. Zool. Station zu Neapel, Bd. 6, pp. 44–92.

Giacomini, E. 1900 a Sulla Struttrua dells branchiedei Petromyzonti. Monit. Zool. Ital., Anno 11, Suppl. 9–10.

1900 b Ibid. (cited from Oppel '05).

Goette, A. 1875 Die Entwickelungsgeschichte der Unke. Leipzig (cited from Hammar, '10).

1890 Entwickelungsgeschichte des Flussneumauges. Hamburg and Leipzig.

Mollier, S. 1906 Die Entwickelung von Blut und Gefassen. In Hertwig's Handbuch der vegl. u. exp. Entwickelungsgeschichte der Wirbeltiere, Jena.

Mozejko 1910 Über die Injektion des Vascularsystems von Petromyzon fluviatalis. Zeitschr. f. wiss. Mikrosk., Bd. 27.

1911 Über den Bau und den morphologischen Wert des Vascularsystem der Petromyzon. Anat. Anz., Bd. 40.

Rabl, C. 1886 Zur Bildungsgeschichte des Halses. Prager Med. Woch., Bd. 11, p. 52 (cited from Hammar, '10).

Rathke, H. 1827 Bemerkung uber den imeren Bau des Querder (Ammocoetes branchialis) und des Kleinen Neunauges (Petromyzon Planeri (cited from Oppel, '05).

Schaffer, J. 1894 Über die Thymusanlage bei Petromyzon Planeri Sitzungsb. d. K. Akad. d. Wiss. Wien., Bd. 103, p. 3.

1895 a Zur Kenntnis des Histologischen und Anatomischen Baues von Ammocoetes. Anat. anz., Bd. 10, pp. 697–708.

1895 b Über das Epithel des Kiemensarmes von Ammocoetes nebst Bemerkungen uber intraepitheliale drusen. Arch. f. Mikr. Anat., Bd. 45, pp. 294–338.

1906 Berichtigung, die Schilddrüse von Myxine betreffend. Anat. Anz., Bd. 28.

Schneider, A. 1879 Beitr. zur Vergl. Anatomie und Entwickelungsgeschichte der Wirbeltiere, Berlin.

Schultze, M. 1856 Die Entwickelungsgeschichte von Petromyzon Planeri. Naturkundige Verhandel, van d. hollandsche Maatschappij. d. Wetenschappen te Haarlem, II Versam, d. 12, p. 28 (cited from Hammer, '10).

Shipley, A. E. 1887 On some points in the development of Petromyzon fluviatilia. Quar. Journ. of Mict. Sc., vol. 27, pp. 325–371.

Stannius, H. 1854 Handbuch der Anatomie der Wirbelthiere (cited from Schaffer, '94).

Stockard, C. R. 1906 The development of the thyroid gland in Bdellostoma Stouti. Anat. Anz., Bd. 29.

Wheeler 1899 The development of the urogenital organs of the lamprey. Zool. Jahrb., Bd. 13.

Willy, A. 1894 Amphioxus and the ancestry of the vertebrates. New York.

PLATE 1

EXPLANATION OF FIGURES

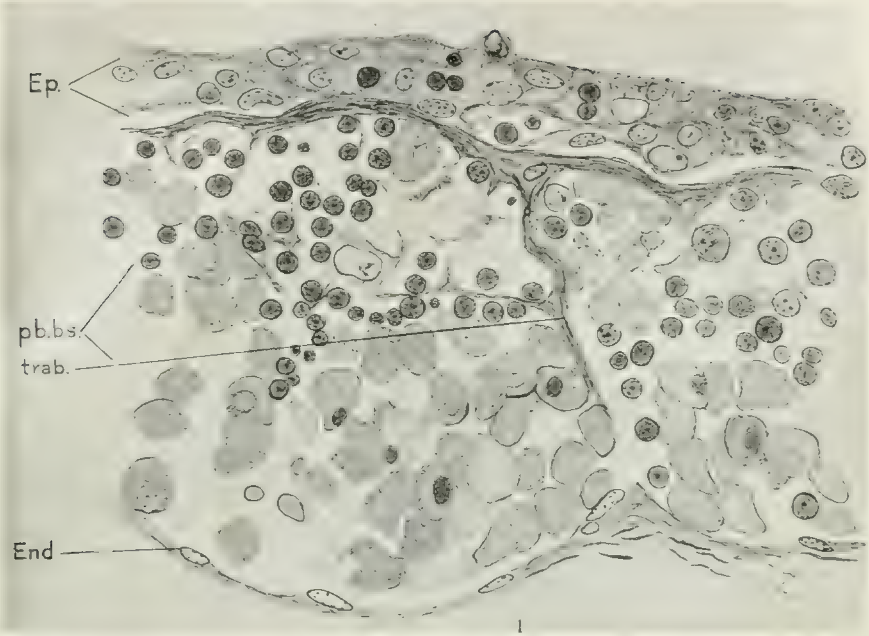
All figures were drawn with the aid of the camera lucida. Higgin's carmine and true blue inks were used to reproduce the colors of the stained sections represented in the colored plate.

1 Lymphoid accumulation in the lateral branchial wall of a 63 mm. larva. *Ep.*, Endodermal epithelium; *End.*, endothelium of blood sinus; *pb.b.s.*, peribranchial blood sinus; *trab.*, connective tissue trabecula in blood sinus. ($\frac{1}{12}$ oil immersion obj., ocular No. 3.)

2 Portion of a frontal section of a 5 mm. larva showing the epithelial placode in the lateral branchial wall. *Ect.*, ectoderm; *End.*, endoderm; *Mes.*, mesenchyma. ($\frac{1}{12}$ oil immer. obj., ocular No. 3.)

3 Epithelial placode in lateral branchial wall of a 9.5 mm. larva. Frontal section. *Am.n.*, amoeboid nuclei; *b.c.*, red blood cells; *lm.*, longitudinal muscle fibers; *tm.*, transverse muscle fibers. ($\frac{1}{12}$ oil immer. obj., ocular No. 3.)

4 Epithelial placode in lateral branchial wall of a 15 mm. larva. Frontal section. *pb.b.s.*, peri-branchial blood sinus; *s.l.*, layer of flat cells forming at surface of placode. ($\frac{1}{12}$ oil immer. obj., ocular No. 3.)



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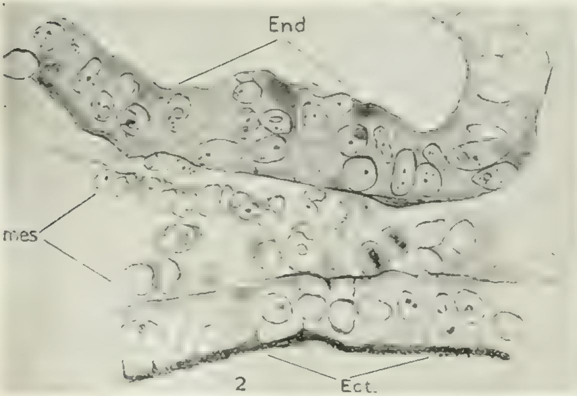

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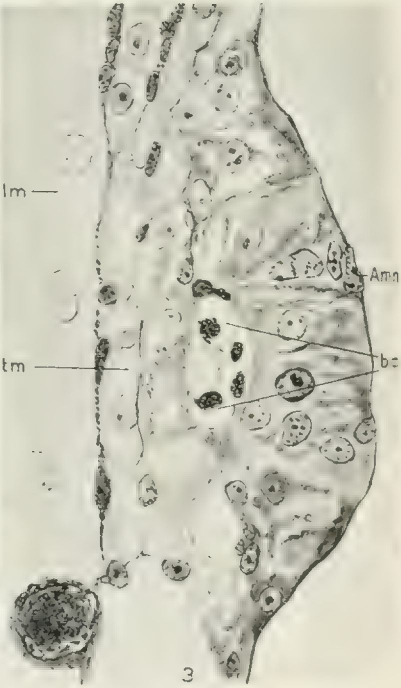

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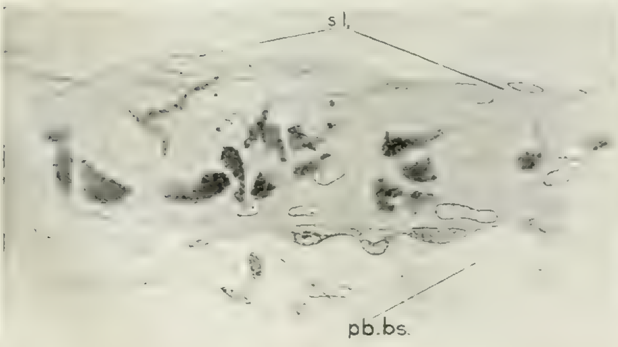

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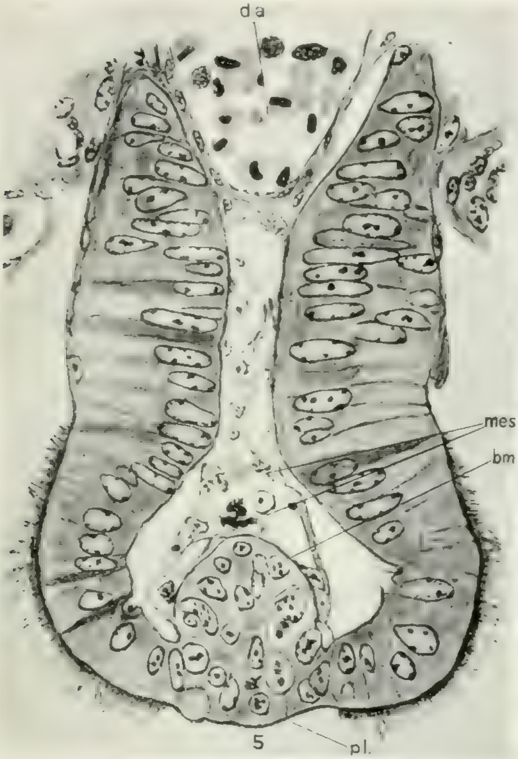
PLATE 2

EXPLANATION OF FIGURES

5 Epipharyngeal ridge containing primitive thymus placode from a 15 mm. larva. *d.a.*, dorsal aorta; *mes.*, mesenchyma containing vascular spaces; *pl.*, primitive thymus placode; *b.m.*, basement membrane separating placode from mesenchyma. ($\frac{1}{12}$ oil immer. obj., ocular No. 3.)

6 Primitive thymus placode in 15 mm. larva. *lm.*, lymphocyte; *mes.*, mesenchyma; *v.*, vacuoles containing protoplasmic bodies. ($\frac{1}{12}$ oil immer. obj., comp. ocular No. 12.)

7 Primitive thymus placode in a 31 mm. larva (ventral surface to the left). *ep.*, nuclei derived from the placode; *c.n.*, connective tissue nuclei; *b.m.*, remains of basement membrane. ($\frac{1}{12}$ oil immer. obj., ocular No. 3.)



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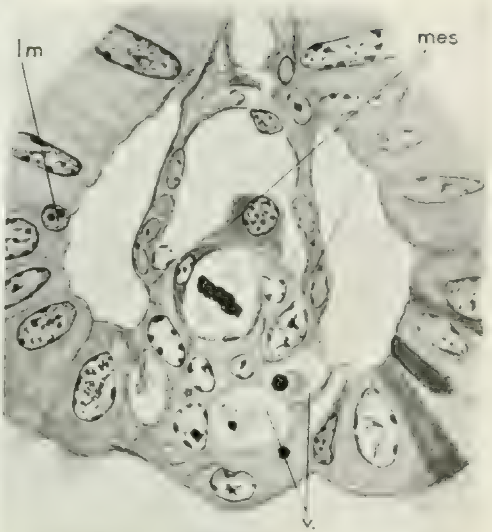

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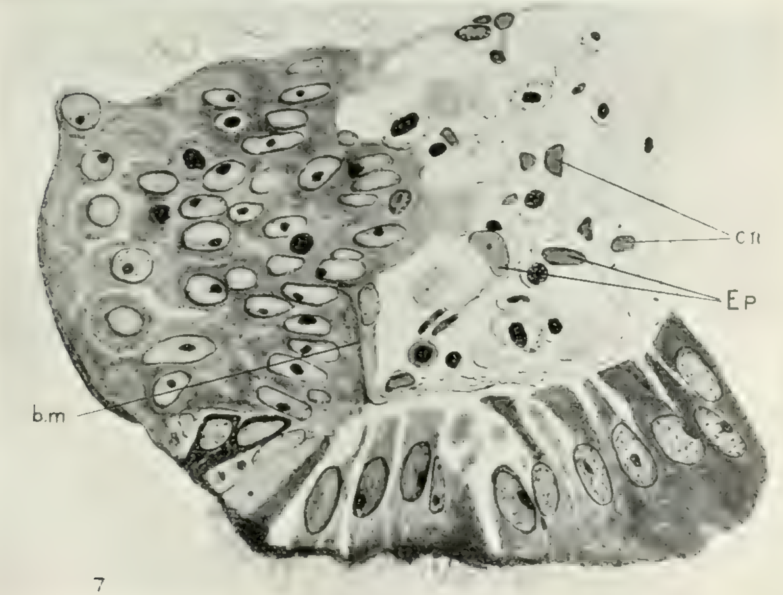

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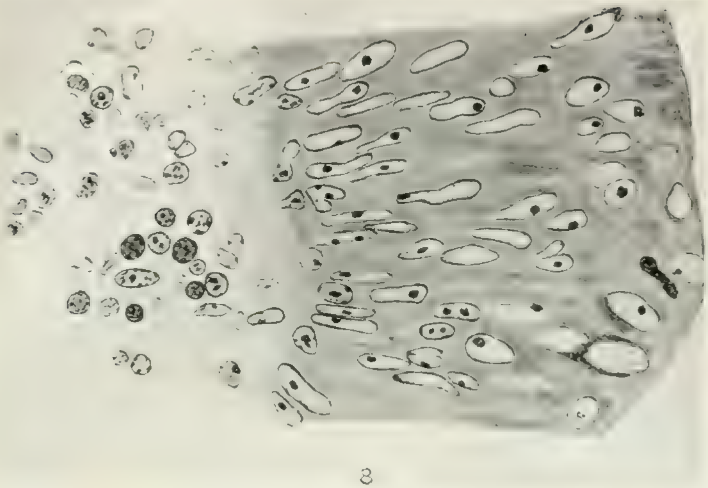
PLATE 3

EXPLANATION OF FIGURES

8 Primitive thymus placode in a 44 mm. larva. Ventral surface to the right. ($\frac{1}{12}$ oil immer. obj., ocular No. 3.)

9 Portion of primitive thymus placode in a 120 mm. larva. *b.m.*, basement membrane; *x.*, cells which are apparently red blood cells held in the connective tissue. ($\frac{1}{12}$ oil immer. obj., ocular No. 3.)



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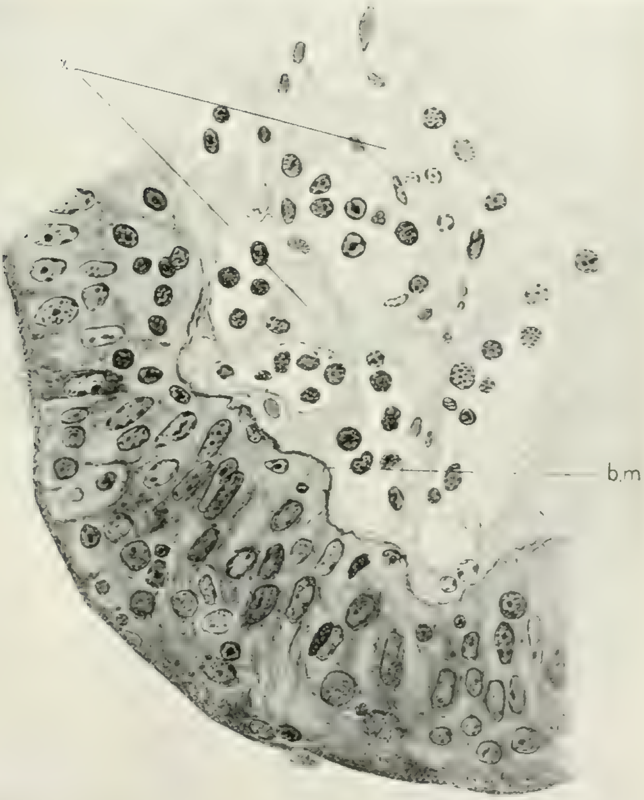

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PLATE 4

EXPLANATION OF FIGURES

10 A small portion of a primitive thymus placode and the connective tissue in relation to it in a 31 mm. larva. The lettering *a* to *j* shows the line of transition from the typical epithelial nucleus (*a*) to the completed lymphocyte-like cell (*j*). ($\frac{1}{12}$ oil immer. obj., comp. ocular No. 12.)

11 Transforming cells from primitive thymus placode of a 31 mm. larva. Cells *g* and *h* (not represented in fig. 10), complete the series of transforming cells shown in figure 10. *c.t.c.*, connective tissue nucleus. ($\frac{1}{12}$ oil immer. obj., comp. ocular No. 3.)

12 Portion of the epithelium of a gill in a 31 mm. larva showing the migration of an epithelial cell (*a*) into a blood vessel. Cells in the left part of the figure (*a* to *h*) show various stages of transformation of the epithelial cell to lymphocyte. *ery.*, red blood cells; *i*, blood cell in vessel in which the nucleus appears to be dividing; *j.*, blood cell in vessel in which there are two nuclei. ($\frac{1}{12}$ oil immer. obj., comp. ocular No. 12.)

13 Epithelial cell beginning migration into blood vessel. ($\frac{1}{12}$ oil immer. obj., comp. ocular No. 12.)

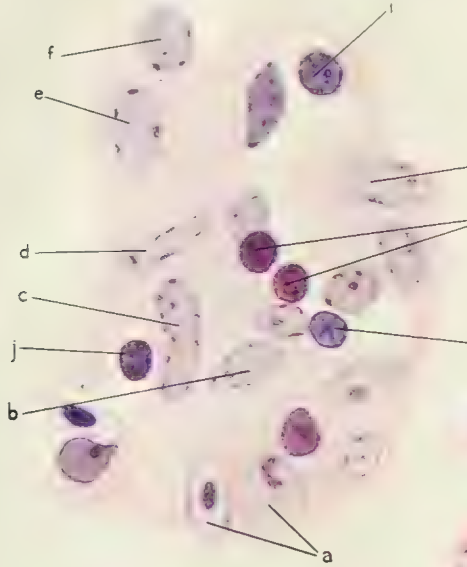
14 Nucleus in primitive thymus placode showing phagocytic properties. ($\frac{1}{12}$ oil immer. obj., comp. ocular No. 12.)

15 Nucleus in primitive thymus placode dividing by simple fission. ($\frac{1}{12}$ oil immer. obj., comp. ocular No. 12.)

16 Epithelial cell found in a connective tissue space in dorsal part of epipharyngeal ridge. Nucleus beginning to divide. ($\frac{1}{12}$ oil immer. obj., comp. ocular No. 12.)

17 Cell found in a connective tissue space in dorsal part of epipharyngeal ridge. Two nuclei in the cell which still retains epithelial characters. ($\frac{1}{12}$ oil immer. obj., comp. ocular No. 12.)

18 Connective tissue nucleus dividing by simple fission. ($\frac{1}{12}$ oil immer. obj., comp. ocular No. 12.)



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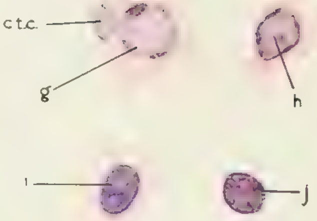

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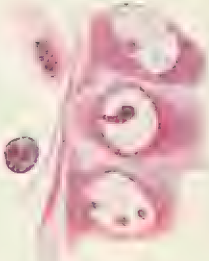

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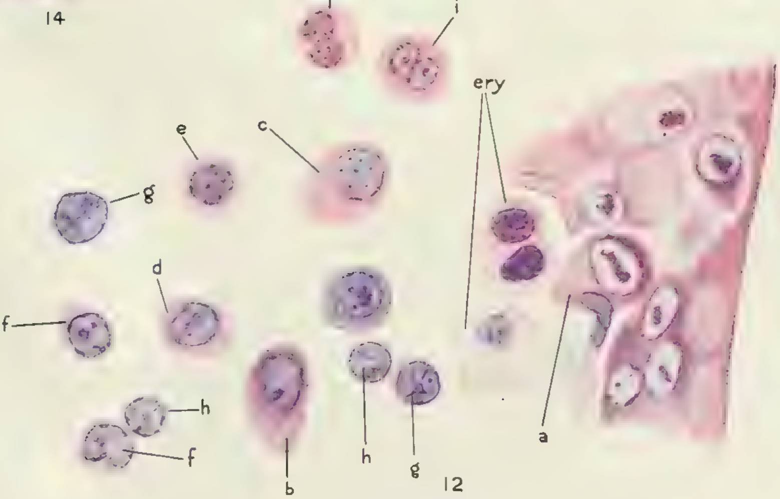

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